rejection. The Examiner has rejected claims 1-9 under 35 U.S.C. 103(a) as being unpatentable over *Yu*. As a basis for this rejection, the Examiner has stated that "the Yu patent publication discloses 'zinc-charged fetuin' which is apoptotic to cancer cells made by the addition of zinc acetate following the isolation of bovine fetal fetuin purified by the modified Spiro method; which 'zinc-charged fetuin' appears to be within the scope of the 'supercharged zinc fetuin' presently claimed." The Examiner further states that *Yu* teaches that fetuin produced by Sigma lacks apoptotic activity due to the use of EDTA and that zinc is the critical ion necessary for apoptotic activity. Accordingly, the Examiner feels that it would be obvious to one skilled in the art to remove other ions with EDTA and then incubate the fetuin with zinc.

Applicant respectfully traverses the Examiner's rejection because Dr. Yu was under an obligation to assign over her rights associated with zinc-charged fetuin to Ambryx Biotechnology, Inc., which is the same entity to which the present inventor has an obligation to assign his inventions.

Pursuant to 35 U.S.C. 103(c), subject matter that is developed by another person, which qualifies under 102(e) as prior art shall not preclude patentability where the subject matter and the claimed invention were, at the time the invention was made, subject to an obligation of assignment to the same

person. As previously submitted, at the time the invention was made, Dr. Yu was under an obligation to assign it over to Ambryx Biotechnology, Inc. Further evidence of this is found in the two attached published articles.

The two articles published by Dr. Tsai and Dr. Yu disclose the identical invention stated in Dr. Yu's application concerning zinc-charged fetuin. The two articles state that "[t]he fetal fetuin isolated from fetal bovine serum by th modified Spiro method was incubated with high concentration of zinc acetate (0.25M) at room temperature for 1 h. The unbound metal ion was then removed by repetitive concentration through a molecular sieve against 20 volumes of PBS repeated four times." *See* Section 2.6, Fetal Fetuin Selectively Induces Apoptosis In Cancer Cell Lines And Shows Anti-Cancer Activity In Tumor Animal Models, Cancer Letters, 166 173-184 (2001) and page 1841, Embryonic Apoptosis-inducing Proteins Exhibited Anticancer Activity In Vitro and In Vivo, Anticancer Research, 21: 1839-1856 (2001). This is precisely what is disclosed in Dr. Yu's application. Dr. Yu's application states "[f]etuin purified by modified Spiro method was further incubated with Zinc Acetate ... (0.25M) at room temperature for 1 hr. Free ions were removed by repetitive concentration against 20 volumes of PBS three times." *See* paragraph 67.

Furthermore, both articles also disclose the importance of zinc to fetuin. In fact, section 3.3 of the article in Cancer Letters as well as section 4.3 of the article in Anticancer Research are both titled "Zinc is an important factor for the apoptosis-inducing activity of fetuin", and both of these sections are substantially indistinguishable from Dr. Yu's disclosure in paragraph 67.

In addition to divulging the identical invention as that disclosed by Dr. Yu, the two articles clearly list, on the first page, Ambryx Biotechnology, Inc. as the company under which these inventions were created and published. This evidence, taken in conjunction with the assignment submitted in the previous response, demonstrate that at the time the invention disclosed in the Yu reference was made, it was subject to an obligation of assignment to Ambryx. Accordingly, pursuant to 35 U.S.C. 103(c), the Yu reference is not prior art against the present claims because both inventors had a duty to assign their inventions to a common entity, Ambryx Biotechnology, Inc.

II. Conclusion.

Based on this supplemental response, along with the response of October 21, 2003, it is Applicant's position that this application is in a condition for immediate allowance, and such action is respectfully requested. If the Examiner believes that a telephone or other conference would b of value in

expediting the prosecution of the present application, enabling an Examiner's amendment or other meaningful discussion of the case, Applicant invites the Examiner to contact Applicant's representative at Trojan Law Offices, 310-777-8399.

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Respectfully submitted,
Trojan Law Offices
By: _____
Roy A. Kim

Date: October 27, 2003

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Fetal fetuin selectively induces apoptosis in cancer cell lines and shows anti-cancer activity in tumor animal models

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Abstract

An apoptosis-inducing protein with molecular weight of 60 kDa has been purified from fetal bovine serum. The N-terminal amino acid sequence of this protein (e.g. I-P-L-D-P-V-A-G-Y-K) reveals that it is bovine fetuin, a fetal protein functioning to control embryogenesis. The apoptosis-inducing activity of fetuin is totally dependent on zinc. Depletion of zinc ion from fetuin or substitution of zinc ion by barium ion completely abolished the apoptosis-inducing activity of fetuin. Interestingly, while the fetuin isolated from fetal serum selectively induces apoptosis in cancer without affecting normal cells, the fetuin isolated from mature serum is completely inactive. This suggests that the biological activity of fetuin is under developmental regulation. In vivo, tumor animal model studies showed that fetuin enhanced survival by up to 141% in P388 leukemia animal model in mice. Fetuin was also found to inhibit prostate cancer formation in a PC-3 prostate cancer model in mice. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Embryo; Fetuin; Apoptosis; Anticancer

1. Introduction

Apoptosis (programmed cell death), in contrast to the cell death caused by cell injury (necrosis), is an active process of gene-directed cellular self-destruction that serves a biologically meaningful function [1]. Apoptosis plays an important role in the human body from the early stages of embryonic development to the inevitable decline associated with old age. In the embryo, cell death is as essential as cell division and cell differentiation in properly regulating cell populations, organ formation and overall body shape. In normal adult tissue, apoptosis occurs continuously in slowly proliferating cell populations such as hepatic [2,3] and adrenal cortical epithelium [4] and in rapidly proliferating populations such as intestinal crypt epithelium [5] and differentiating spermatogonia [6].

We have been interested in the phenomenon that apoptosis is a highly active biological event in the embryo. Glucksmann has enumerated 74 separate examples of embryonic cell death in 1950 [7]. The factor(s) that induce(s) the numerous apoptosis events in the embryo is unclear. Inspired by the findings reported by Gerschenson's group that a heat- and trypsin-sensitive, apoptosis-inducing factor was secreted by primary culture of rabbit endometrial cells [8], we hypothesized that certain embryonic tissues may secret soluble proteins that induce the highly activ apoptotic event in the embryo. To test this hypothesis, we tested whether fetal serum contains apoptosis-

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inducing activity. This approach is based on the assumption that the soluble apoptosis-inducing proteins secreted by the embryo may control cell death by acting in a paracrine, endocrine or autocrine manner; hence, fetal serum may contain such apoptosis-inducing proteins. As we expected, an apoptosis-inducing protein was found in fetal bovine serum. This protein has been purified, sequenced and identified to be bovine fetuin, a fetal protein that has been reported to play an important role in embryonic development [9,10]. The fetuin-induced apoptosis was found to be Zn^{2+}-dependent. More interestingly, we found that only the fetuin isolated from fetal serum contains apoptosis-inducing activity. The fetuin isolated from mature serum is completely inactive. Fetuin selectively induced apoptosis in cancer without having an effect in normal cell lines in a very rapid time course. Based on the selectivity, potency and rapidity in inducing apoptosis, fetuin may be developed as an anticancer agent. This notion is further supported by in vivo animal studies, which showed the anticancer effect of fetuin in mice P388 leukemia and PC-3 prostate cancer models.

2. Materials and methods

2.1. Cell cultures

The human promyelocyte leukemia cell line, HL-60 cells (CCL240; ATCC, Rockville, MD) were grown in RPMI 1640 (containing 20% fetal calf serum). The human metastatic prostate adenocarcinoma cell line, LNCaP (CRL 1740; ATCC) was grown in RPMI 1640 medium containing 15% FBS and penicillin–streptomycin (10,000 units/ml). The human colon carcinoma cell line, Colo 205 (CCL 222; ATCC) was grown in RPMI 1640 medium containing 10% FBS, sodium pyruvate, sodium bicarbonate and penicillin–streptomycin (10,000 units/ml). The human prostate adenocarcinoma cell line, PC-3 (CRL 1435; ATCC) was grown in HAM's F-12 medium containing 10% FBS and penicillin–streptomycin (10,000 units/ml). The human breast adenocarcinoma cell line, MCF-7 (HTB 22; ATCC) was grown in Eagle's minimal essential medium (MEM) containing 10% FBS, sodium pyruvate, non-essential amino acid, 1 mM bovine insulin and penicillin–streptomycin (10,000 units/ml). The human lung carcinoma cell line, Calu-1 (HTB 54; ATCC) was grown in McCoy's 5a medium containing 10% FBS and penicillin–streptomycin (10,000 units/ml). The human normal lung fibroblast cell line, WI-38 (CCL 75; ATCC) was cultured in Eagle's MEM containing 10% FBS, non-essential amino acid and penicillin–streptomycin (10,000 units/ml). The human normal lung cell line, CCD-39Lu (CRL 1498; ATCC) was grown in Eagle's MEM containing 10% FBS, Eagle's BSS, non-essential amino acid and penicillin–streptomycin (10,000 units/ml). The human normal colon fibroblast, CCD-18Co (CRL 1459; ATCC) was grown in Eagle's MEM containing 10% FBS, Eagle's BSS, non-essential amino acid and penicillin–streptomycin (10,000 units/ml).

2.2. Apoptosis assays

Hoechst dye staining and MTT assays were used for apoptosis assay. The assays were performed in the presence of 5–10% FBS as described below.

2.2.1. Hoechst dye assay

Cultured cells (5,000 cells) were seeded in 10 μl growth medium containing 10–20% fetal bovine serum at 37°C, 5% CO_2 in microtray plates (25 μl wells, Robbins Scientific Corp.). The tested sample (10 μl), after the buffer was changed to PBS, was added 12 h after cells were seeded. After incubation of the tested sample with cells for 15 h, 2 μl of Hoechst 33258 (0.1 μg/ml in PBS) was added. Two hours later, cells that were stained with Hoechst dye were examined under a fluorescence microscope. The nuclei of apoptotic cells showing DNA condensation and fragmentation are easily identified by Hoechst dye staining. The percentage of apoptotic cells is calculated using the following equation: % Apoptotic cell = Number of cells with DNA condensation and/or fragmentation/Total cell number.

2.2.2. MTT assay

The MTT assay has been widely used as a cytotoxicity assay. Cells grown in 96-well microtiter plate (200 μl containing 10^5 cells) were incubated with Thiazolyl Blue (MTT, 125 μg/ml) for 4 h at 37°C. The reaction may be stopped earlier if the color develops rapidly (this depends on the cells in question). At

the end of the incubation period, the plate was centrifuged at 1200 rev./min for 5 min. The supernatant was removed, leaving 50 μl in each well to ensure that no cells are aspirated. To this 100 μl 0.04 M HCl in isopropanol was added to each well. This solution solubilized the formazan product and produced a homogeneous solution suitable for measurement of optical density. After a 5-min incubation at room temperature to ensure that all the crystals were dissolved, the plate was read on a microELISA reader using a test wavelength of 595 nm. The advantage (and disadvantage) of this assay is that it is quantitative but not qualitative. Any cell death, apoptotic or necrotic, may be detected by this method. This assay is used complementarily with the Hoechst dye assay.

2.3. Preparation of genomic DNA for agarose gel analysis of DNA fragmentation

Cultured cells (10×10^6) were digested by incubating with digestion buffer (10 mM Tris–HCl (pH 8.0) containing 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate and 0.2 mg/ml proteinase K) at 50°C for 18 h. Cell lysate was then extracted twice with phenol following by twice with phenol/chloroform (1:1) and twice with chloroform/isoamyl alcohol (24:1). Genomic DNA was extracted from the aqueous layer by cold 100% ethanol after proteins were removed by phenol and chloroform extraction. DNA pellet was rinsed for 5 min with 70% cold ethanol. Ethanol was decanted or aspired and pellet was dried completely. DNA pellet was then resuspended in TE buffer and digested with 0.2 mg/ml DNAse-free RNAse. Genomic DNA was developed by 2% agarose gel at 55 V for 5 h. Gel was stained by ethidium bromide (0.4 μg/ml) for at least 30 min.

2.4. Purification of apoptosis-inducing protein from fetal bovine serum

LNCaP cells were routinely used in the assays for the purification of apoptosis-inducing protein present in fetal bovine serum. The apoptosis-inducing protein was purified by the following procedures:

2.4.1. Ammonium sulfate precipitation

Fetal bovine serum (100 ml, BioWhittaker) was treated with 80% saturated ammonium sulfate (56.1 g). Precipitate was collected by centrifugation and dissolved in 100 ml Tris–HCl (20 mM, pH 7.5). Ammonium sulfate was removed by dialysis against the same buffer overnight, insoluble precipitate was discarded after centrifugation. Protein solution was concentrated to 10 ml.

2.4.2. Hydroxylapatite treatment

After removal of ammonium sulfate by dialysis in 10 mM Tris–HCl (pH 7.5), the dissolved proteins were incubated with hydroxylapatite gel (Bio-Gel HTP gel, Bio-Rad) for 1 h. After removing HTP gel by centrifugation, an activity inducing apoptosis in LNCaP cells was found in the supernatant which was then further treated with heparin agarose gel.

2.4.3. Heparin agarose treatment

The supernatant from Section 2.4.2 was further incubated with heparin agarose (Sigma) for 1 h. After removing heparin gel by centrifugation, the activity inducing apoptosis in LNCaP cells was found in the supernatant.

2.4.4. Reverse phase chromatography

The apoptosis-inducing protein presents in the supernatant of heparin agarose in Section 2.4.3 was further purified by a reverse phase chromatography. Protein sample was concentrated to 1 ml. One milliliter of methanol containing 0.05% trifluoroacetic acid (TFA) was added. A large amount of proteins was precipitated by this treatment, whereas the apoptosis-inducing activity remained in the supernatant. The supernatant was then applied to a reverse phase RP-4 column (Micra Scientific Inc.) and developed by a linear gradient consisting by solution A (H_2O, 0.05% TFA) and solution B (methanol, 0.05% TFA). The linear gradient was created by increasing solution B from 0 to 100% in solution A in 10 min (20 ml elution volume) and thereafter, the column was eluted with 100% solution for 5 min. The purity of the isolated apoptosis-inducing protein was determined by a SDS–polyacrylamide gel stained with silver staining. A single protein band with molecular weight of 60 kDa was obtained (Fig. 2).

2.5. Purification of fetuin from fetal bovine serum

Bovine fetuin was prepared by a modified Spiro method [11].

(1) Two hundred milliliters of 0.05 M zinc acetate containing 30% (v/v) ethanol was added to 100 ml of fetal bovine serum (FBS): pH was adjusted to 6.4 by 1 M NH_4OH-NH_4Cl, and left to stand for 15 h at −5°C. (2) The supernatant was collected by centrifugation, 1.0 M barium acetate and 95% ethanol added to give 0.03 M barium acetate, 25% ethanol. This was left to stand for 2 h at −5°C. (3) The supernatant was collected by centrifugation, and 95% ethanol added to give 40% ethanol. This was left to stand for 16 h at −10°C. (4) The precipitate was collected by centrifugation. The pellet was dissolved using phosphate-buffered saline (PBS). Free zinc acetate and barium acetate were removed by repetitive concentration through a molecular sieve against 20 volumes of PBS for repeated four times. The modified Spiro method differs from the original Spiro method in that no extensive dialysis nor chelating agent (trisodium citrate) treatment was used in our preparation. The purified fetuin showed a single protein band with apparent molecular weight of 60 kDa on SDS–polyacrylamide gel electrophoresis.

2.6. Preparation of Zn^{2+}-charged fetal fetuin

The fetal fetuin isolated from fetal bovine serum by the modified Spiro method was incubated with high concentration of zinc acetate (0.25 M) at room temperature for 1 h. The unbound metal ion was then removed by repetitive concentration through a molecular sieve against 20 volumes of PBS repeated four times.

2.7. Preparation of mature fetuin

The mature fetuin was prepared by the same method for fetal fetuin preparation, except that adult bovine serum, instead of fetal serum, was used for the preparation.

2.8. In vivo tumor animal study (P388 leukemia model)

Forty DBA/2 female mice (17–20 g) kept on a standard diet and water ad libitum were inoculated with tumor cell line P388D1 (ATCC; CCL 46). Freshly harvested tumor cells were diluted with saline. One million cells were injected intraperitoneally. The mice were randomly segregated into groups of ten and housed in shoebox cages. Zn^{2+}-charged fetal fetuin was intraperitoneally injected into group I (1 mg/kg), group II (10 mg/kg) and group III (100 mg/kg). A control group (group IV) was included and injected with saline on the days that the tested animals were treated. The injection were continued for 10 days. Mortalities were recorded daily. Results are expressed as the percentage increase in life span (ILS): ILS = 100 × (Median Life Span Treated − Median Life Span Controlled)/Life Span Controlled.

2.9. In vivo tumor animal study (PC-3 prostate cancer model)

Male nude mice (25–30 g)were used in this study. The prostate adenocarcinoma cell; PC-3 (2 million cells) were injected on the upper half of the dorsal thorax of the mice. The tumors were allowed to grow for 6 weeks. The mice in the control group (no treatment) received 0.1 ml saline intraperitoneally for 5 days and the mice in the treated group received 50 mg/kg fetuin. Treatment started the day after tumor inoculation for 5 days. Six weeks after the inoculation of tumor, mice were killed and tumors were removed and weighed.

3. Results

3.1. Fetal bovine serum contains apoptosis-inducing activity in LNCaP cells

LNCaP cells grown in 10 μl RPMI 1640 medium containing 15% FBS were incubated with the control buffer or the fetal bovine serum extract for 15 h and then stained with Hoechst dye for 2 h. We found that the nuclei of the cells that had been incubated with control buffer (BSA in PBS and RPMI containing 7.5% FBS) were normal and healthy. However, the nuclei of the LNCaP cells that had been incubated with the fetal serum extract (PBS and RPMI containing 7.5% FBS) showed the characteristics of apoptosis. First, the fetal serum extract causes the condensation of nucleus, which is demonstrated by the more intense fluorescent light compared with the control nucleus. Secondly, the nuclear condensation is accompanied by the fragmentation of DNA, which is demonstrated by the breakage of nucleus (data not shown). To further biochemically demonstrate the

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induction of apoptosis by fetal serum extract, genomic DNA was extracted from the LNCaP cells and run on a 2% agarose DNA gel. As shown in Fig. 1, a so-called DNA ladder was observed in the experimental group, suggesting the induction of apoptosis in LNCaP cells by fetal serum extract. The apoptosis-inducing activity in fetal bovine serum was found to be sensitive to proteinase K, which suggests the protein nature of this activity (data not shown).

Fig. 1. Agarose gel (2%) electrophoresis of DNA extracted from cultures of LNCaP cells incubated with fetal bovine serum extract. Genomic DNA prepared from LNCaP cells (10^6 cells) was developed by 2% agarose gel at 55 Vs for 5 h. Gel was stained by ethidium bromide (0.4 g/ml) for at least 30 min. (A) Control; (B) fetal bovine serum extract.

LNCaP cells were then routinely used in the assays for the purification of the apoptosis-inducing protein present in fetal bovine serum. Using the procedure as described in Section 2, the apoptosis-inducing protein was purified to homogeneity from fetal bovine serum. As shown in Fig. 2, a single protein band with a molecular weight of 60 kDa was obtained. this protein band was then transferred to PVDF membrane and the N-terminal amino acid sequence was determined. An amino acid sequence of I-P-L-D-P-V-A-G-Y-K was obtained. GenBank BLAST search showed that this sequence is identical to the N-terminal amino acid sequence of bovine fetuin.

3.2. Fetal fetuin isolated by a modified Spiro method induces apoptosis

To further confirm that bovine fetuin per se contains apoptosis-inducing activity, we tested the fetuin purified from fetal bovine serum by a modified Spiro method. As shown in Fig. 3, the fetuin purified by a modified Spiro method strongly induced apoptosis in certain cancer cell lines such as: LNCaP (human metastatic prostate adenocarcinoma), PC-3 (human prostate adenocarcinoma), HL-60 (human promyelocyte leukemia), MCF-7 (human breast adenocarcinoma), Colo 205 (human colon carcinoma) and Calu-1 (human lung carcinoma). Non-cancerous, normal cell lines such as WI-38 (human normal lung fibroblast), CCD-39Lu (human normal lung) and CCD-18Co (human normal colon fibroblast), on the other hand, are not affected by fetuin. The morphological demonstration of the induction of apoptosis in cancer cell lines (LNCaP, HL-60 and Colo 205) by fetuin is shown in Fig. 4. The incubation of fetuin (2 M) results in the condensation and fragmentation of DNA, which are demonstrated by a more intense fluorescence and breakage of nuclei, respectively. Normal cell line, WI-38, CCD-39Lu and CCD-18Co, as shown in Fig. 4, are not affected by the incubation of fetuin.

3.3. Zn^{2+} is an important factor for the apoptosis-inducing activity of fetuin

The Zn^{2+}-charged or Ba^{2+}-charged fetuin prepared as described in Section 2 were tested in LNCaP cells. As shown in Fig. 5, Zn^{2+}-charged fetuin strongly and rapidly induced apoptosis with an activity higher than

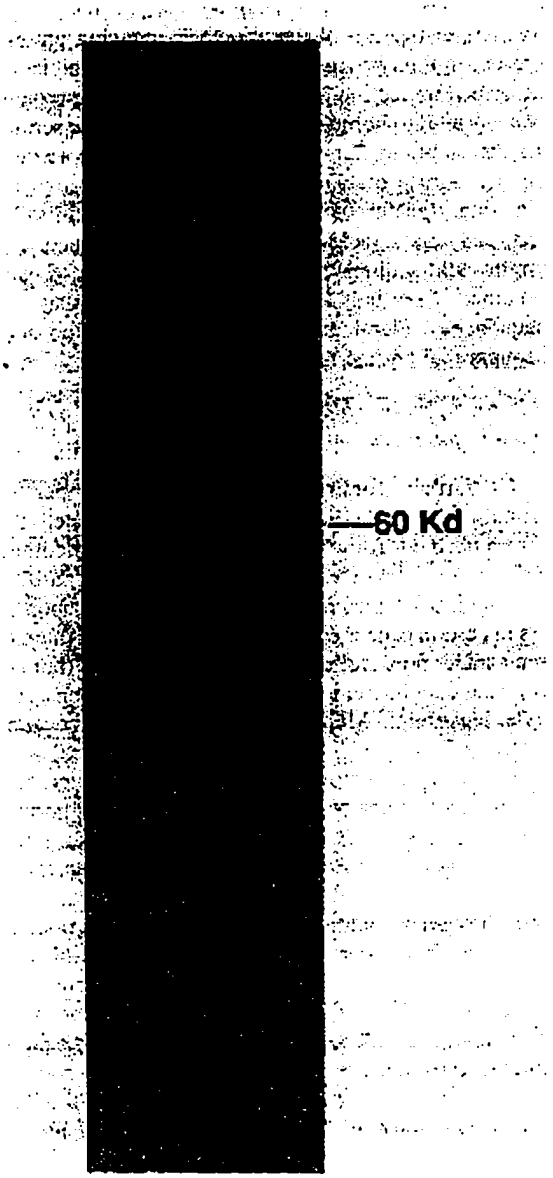


Fig. 2. SDS-polyacrylamide gel electrophoresis of purified apoptosis-inducing protein from fetal bovine serum. The apoptosis-inducing protein was purified from fetal bovine serum as described in Section 2. Protein (20 μl) was fractionated by 4–20% gradient gel and silver stained. In a separate run, about 200 ng of protein sample was run on gel and stained with Coomassie blue. The protein band was then transferred to a PVDF membrane for N-terminal amino acid sequence determination.

that of the originally uncharged fetuin. The induction of apoptosis in LNCaP cells by Zn^{2+}-charged fetuin so strong and rapid that almost 100% of LNCaP cells was under apoptosis in 4 h. On the other hand, Ba^{2+} charged fetuin failed to show any activity inducing apoptosis in LNCaP cells. The same result is obtained in an assay using Colo 205 cells. This result suggests that Zn^{2+} is necessary for fetuin to induce apoptosis. Substitution of Zn^{2+} by Ba^{2+} completely abolish the apoptosis-inducing activity of fetuin. Zn^{2+}-charged fetuin induced apoptosis in various cancer cell lines in a dose-dependent manner. The LD_{50} of fetuin induced apoptosis in each cell line is shown in Table 1. The relative sensitivity of cancer cell lines to fetuin is: LNCaP = PC-3 > Calu-1 > HL-60 > Colo205 > MCF-7 > Hep G2, whereas the three normal cell lines, CCD-39Lu, CCD-18Co and WI-38, seem to be insensitive to Zn^{2+}-charge fetuin. Fig. 6 shows the time course of the induction of apoptosis in tumor cells by Zn^{2+}-charged fetuin. We found that fetuin rapidly induced apoptosis in cancer cell lines. Zn^{2+}-charged fetuin caused cell shrinkage and DNA condensation in LNCaP, PC-3, Colo 205, HL-60 and Ca lu-1 cells in as short a time as 15 min (Fig. 6).

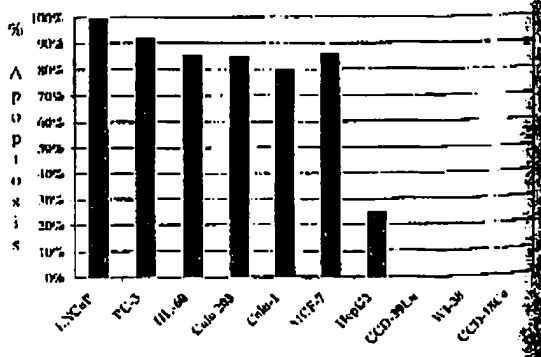
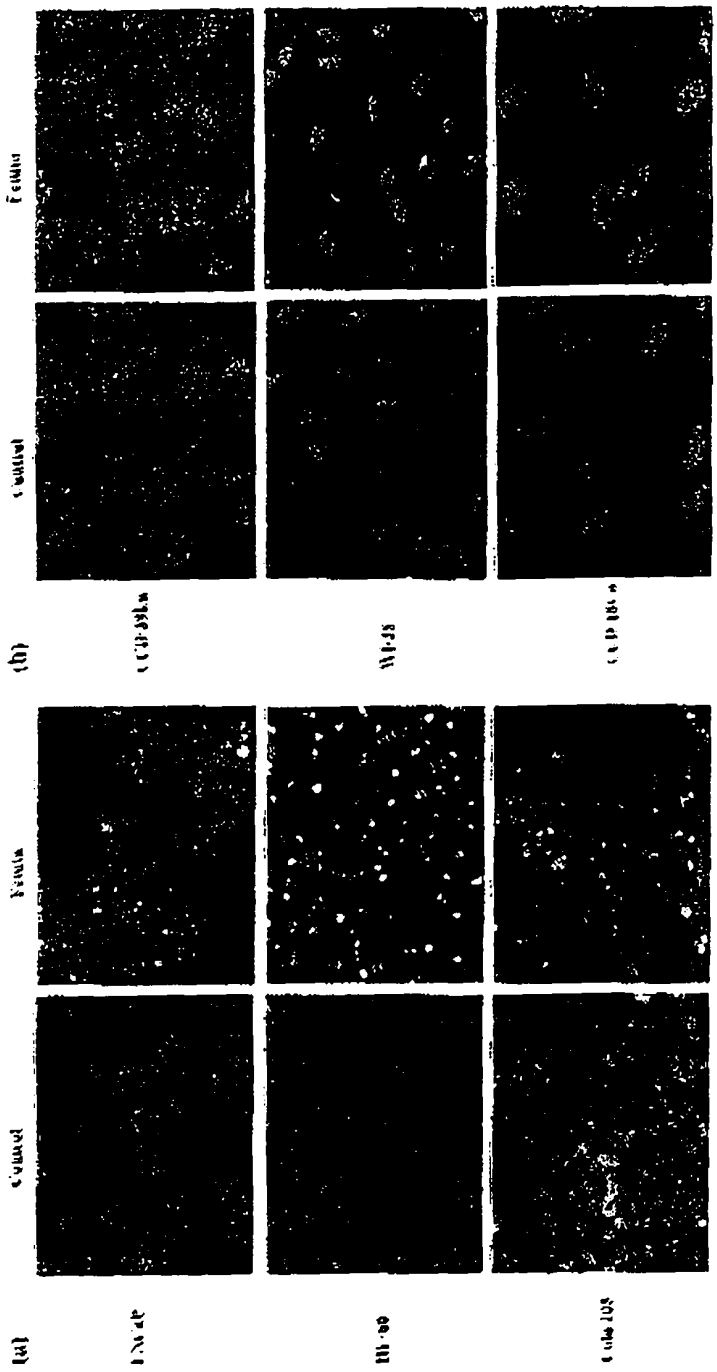


Fig. 3. Fetuin induced apoptosis in various cancer cell lines. Fetuin was purified by a modified Spiro method as described in Section 2. After the buffer was changed to PBS by repetitive concentration by a molecule sieve, fetuin (250 μg/ml) was incubated with cultured cells for 10 h. Cells were then stained by Hoechst 33258 (0.1 μg/ml) for 2 h and inspected under a fluorescence microscope. Each point represents the average of three assays. Trace amount of zinc acetate and barium acetate may remain in the fetuin preparation. All of the cell lines are not affected by zinc acetate or barium acetate alone at all the concentrations tested (up to 66 M).



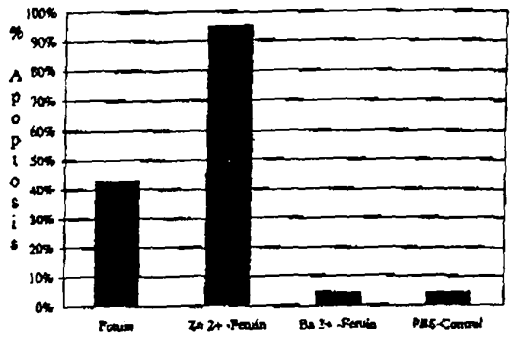


Fig. 5. Induction of apoptosis in LNCaP cells by fetuin. Zn^{2+}-charged fetuin and Ba^{2+}-charged fetuin. The fetuin (5 μM) purified by a modified Spiro method was incubated with zinc acetate or barium acetate (0.25 M) at room temperature for 1 h. Free ions were removed by repetitive concentration against 20 volumes of PBS three times. Fetuin, Zn^{2+}-charged fetuin, Ba^{2+}-charged fetuin and PBS were then separately incubated with LNCaP cells for 4 h. Percentage of cells under apoptosis was determined by Hoechst dye staining and confirmed by MTT assay. Each point represents the average of two assays.

3.4. Fetal, but not mature fetuin contains apoptosis-inducing activity

Fetal fetuin and mature fetuin isolated from fetal bovine serum and mature bovine serum, respectively, were tested on LNCaP cells. We found that the fetuin isolated from fetal bovine serum strongly induced apoptosis in LNCaP cells, whereas the fetuin (at the same protein concentration) isolated from mature bovine serum showed no activity inducing apoptosis (Fig. 7). This result may suggest the molecular change of fetuin during development.

Table 1
LD_{50} of fetuin-induced apoptosis in various cell lines[a]

Cell line	LD_{50} (μM)
LNCaP	1
PC-3	1
Colo 205	5
Calu-1	4
HL-60	5
MCF-7	8
Hep G2	20
CCD-39Lu	>100
CCD-18Co	>100
WI-38	>100

[a] Various concentrations of Zn^{2+}-charged fetuin prepared as described in Fig. 5 were incubated with cell lines for 6 h. Percentage of cells under apoptosis was determined by Hoechst dye staining and confirmed by MTT assay. The concentration of Zn^{2+}-charged fetuin for the induction of 50% of cells under apoptosis (LD_{50}) was determined. Each datum represents the average of three assays.

3.5. Antileukemia effect of fetal fetuin in mice

Table 2 shows the increase of survival of leukemia-bearing mice treated with fetal fetuin. It was found that while 100% of untreated mice (injected with saline) were dead after 24 days, 80% (eight of ten) of mice injected with high dose (100 mg/kg) of fetal fetuin survived after 58 days. This experiment showed that the treatment of fetal fetuin increased the life span of P388 leukemia-bearing mice by 141%.

3.6. Fetuin completely inhibited prostate cancer in mice

As described in Section 2, 6 weeks after the inoculation of tumor, nude mice were killed and tumors were weighed. While tumors (average weight 325 mg) developed in 13 of 13 mice in the control group (treated with saline), none of the nine mice treated with fetuin (50 mg/kg) developed any tumor (Fig. 8).

Fetuin seems to completely inhibit the formation of prostate cancer (PC-3) in this experiment.

4. Discussion

Fetuin was first identified over five decades ago [12]. The present finding is the first report showing that fetuin induces apoptosis. The main reason that the apoptosis-inducing activity of fetuin was not found previously is that the apoptosis-inducing activity of fetuin is largely affected by the method of preparation. For example, fetuin prepared by $Zinc^{2+}$/ethanol precipitation (Spiro method) is able to induce alkaline phosphatase but fails to inhibit trypsin [13], whereas fetuin prepared by the Pedersen method [12] is able to inhibit trypsin without inducing alkaline phosphatase [13,14]. In the present study, we prepared the fetuin that contains apoptosis-inducing activity by a modified Spiro method, which, unlike the original Spiro method, does not use prolonged dialysis or chelating agent such as trisodium citrate. Hence, the fetuin we prepared may remain in a Zn^{2+}-charged form. Furthermore, the

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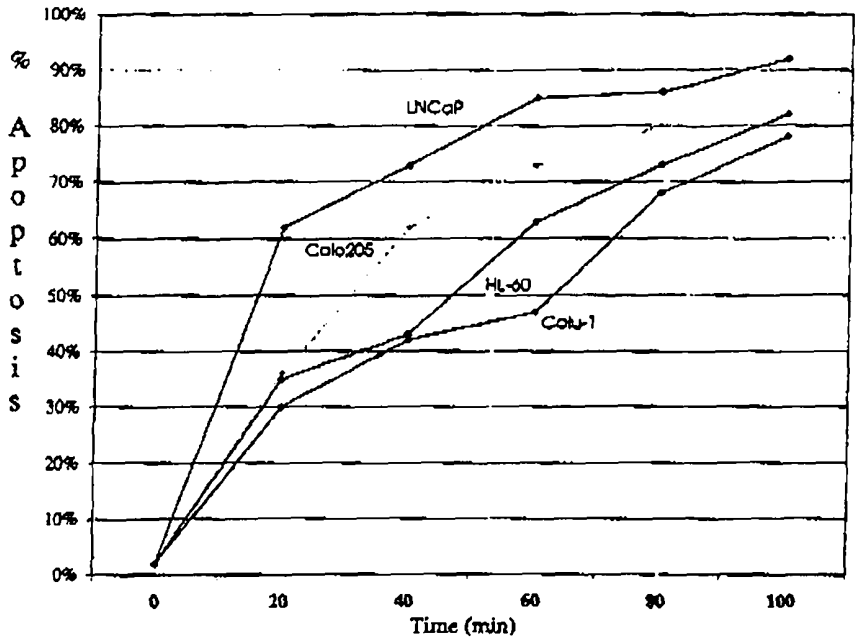

Fig. 6. Time course of the induction of apoptosis in cancer cell lines. Zn^{2+}-charged fetuin (5 μM) prepared as described in Fig. 5 was incubated with cell lines. At various time intervals, cells under apoptosis (cell shrinkage and DNA condensation) were determined by Hoechst dye staining and inspected under a fluorescence microscope. Each point represents the average of two assays.

apoptosis-inducing activity of fetuin we prepared can be further enhanced by incubation with high concentration of zinc ion, whereas incubation of barium completely abolished the apoptosis-inducing activity of fetuin (Fig. 5). Theses results suggest that fetuin induced apoptosis in a Zn^{2+}-dependent manner. The preparation of fetuin by the Pedersen method [12] does not involve the treatment of zinc ion and hence is inactive in inducing apoptosis.

Fetuin is expressed at very high levels throughout the long gestational period of bovine and accounts for up to 50% of the total fetal serum protein [15]. However, the concentration of fetuin in sheep and bovine serum drastically reduce in adult to 1–2% of the fetal level [9]. Although the quantitative change of fetuin during development seems to be obvious, the qualitative change (if any) of fetuin during development is unclear. It is known that an enzyme such as γ-glutamyltransferase changes its structure and activity during development. Depending on the developmental stage, the γ-glutamyltransferase exists as two

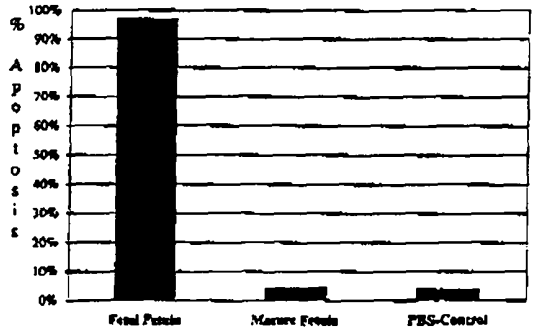

Fig. 7. Fetal, but not mature fetuin contains apoptosis-inducing activity. Fetuins were separately purified from fetal bovine serum or mature bovine serum by a modified Spiro method as described. Equal amount of fetal or mature fetuin (5 μM) was incubated with LNCaP cells for 15 h. Percentage of cells under apoptosis was determined by Hoechst dye staining and confirmed by MTT assay. Each point represents the average of two assays.

Table 2
Effect of fetal fetuin in P388 leukemia animal model[a]

Group	No. of mice	Dose (mg/kg)	Survivors (days)	ILS (%)
I	10	1	1 (31)	29
II	10	10	1 (29)	17.2
III	10	100	8 (58)	141
IV	10	0.5 ml saline	0 (24)	-

[a] Zn^{2+}-charged fetal fetuin was intraperitoneal injected into mice inoculated with P388D1 cells as described in Section 2. The injected doses are: group I (1 mg/kg), group II (10 mg/kg) and group III (100 mg/kg). A control group (group IV) was included and injected with saline on the days that the tested animals were treated. The injection were continued for 10 days. Mortalities were recorded daily. Results are expressed as the percentage increase in life span (ILS): ILS = 100 × (Median Life Span Treated − Median Life Span Controlled)/Life Span Controlled.

different types with different activity: a sialic acid-rich fetal type and a sialic acid-poor adult type [16]. In the present study, we found that fetuin isolated from fetal serum strongly induced apoptosis, whereas the fetuin isolated from mature bovine serum is completely inactive. This result suggests a possible qualitative change of fetuin during development. A preliminary attempt to differentiate fetal fetuin from mature fetuin shows that they behave similarly on hydroxylapatite and anionic exchange chromatographies. However,

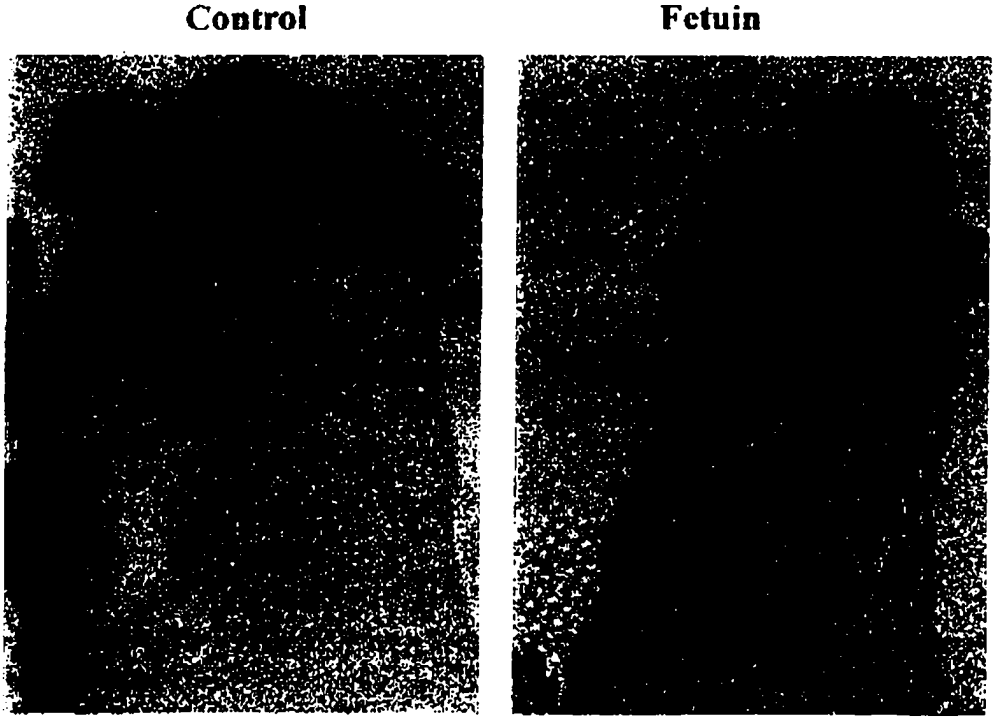

Fig. 8. Fetuin completely inhibited prostate cancer formation in PC-3 prostate cancer model in mice. Male nude mice (25–30 g) were inoculated with PC-3 cells, as described in Section 2. Six weeks after the inoculation of tumor, nude mice were killed and tumors were weighed. While tumors (average weight: 0.28 mg) developed in 13 of 13 mice in the control group (treated with saline), none of the nine mice treated with fetuin (50 mg/kg) developed any tumor.

the elution profiles of fetal and mature fetuins on a Con A–Sepharose chromatography were found to be significantly different (unpublished observation). This observation suggests that a developmental change on the glycosylated state of fetuin, which affects its apoptosis-inducing activity, may occur. The molecular difference between fetal and mature fetuins is currently under investigation.

Fetuins are members of the cystatin superfamily of proteins possessing two tandemly arranged cystatin domains and a third domain rich in proline and glycine. Secondary modifications, *N*-glycosylation, *O*-glycosylation, Ser-phosphorylation, and proteolytic processing have all been described for a variety of fetuins from several species [17–20]. Despite this wealth of information on the structure of fetuins, their biological function is still far from clear. Evidence suggests that fetuin may play an important role in embryonic development; during mouse embryogenesis, fetuin mRNA is expressed in a number of developing tissues and organs, including the heart, lung, kidney, nervous system and liver [9]. In addition, fetuin mRNA is expressed in the developing limb buds of 12-day mouse embryos but not at 16 or 19 days of gestation [9]. Furthermore, immunohistochemical study found that the colloid material (aggregate of dead cells) of developing human pituitary gland contains fetuin throughout the first half of gestation [10]. This finding prompted the suggestion that fetuin is part of a homeostatic system, which controls remodeling and physiological cell death during development [10]. In this regard, our current finding that fetuin contains apoptosis-inducing activity seems to consist with the conclusion made by this immunohistochemical study.

The mechanism by which fetuin induces apoptosis is currently unknown. Fetuin has been found to induce alkaline phosphatase [14], a protein that is able to induce apoptosis [21]. Data indicate that when alkaline phosphatase is expressed in high levels, cells are more likely to die because of apoptosis [21]. Previous data also have shown that in rodents, high levels of alkaline phosphatase expression are found in fibroblasts adjacent to bone and tooth surface where high numbers of apoptotic cells are found [22,23]. These results raise the possibility that induction of alkaline phosphatase may represent a mechanism by which apoptosis is induced by fetuin.

Fetuin-induced apoptosis was found to be highly selectively. We found that Zn^{2+}-charged fetal fetuin selectively induced apoptosis in cancer cell lines without having an effect in normal cell lines. For example, fetuin strongly (>90%) and rapidly (<1 h) induced apoptosis in HT-29 cells (human colon adenocarcinoma) and Calu-1 cells (human lung carcinoma) without having an effect in CCD-18Co cells (human normal colon fibroblast) and CCD-39Lu cells (human normal lung fibroblast). Based on the selectivity, potency and rapidity in inducing apoptosis in cancer cells, fetuin may be developed as an anticancer agent. This notion was further tested on tumor animal model studies. In the P388 leukemia model, we found that fetuin enhanced survival rate by up to 141% in leukemia-bearing mice. In the PC-3 prostate cancer animal model, fetuin seems to completely inhibit the formation of prostate cancer in mice. These results strongly support the notion that fetuin may be developed as an anticancer agent.

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Abstract

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Embryonic Apoptosis-inducing Proteins Exhibited Anticancer Activity *In Vitro* and *In Vivo*

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Abstract. *A 54 Kd apoptosis-inducing protein with novel amino acid sequence has been purified from the conditioned medium of the embryonic cell line, C3H 10T1/2 cells. An apoptosis-inducing protein identified to be fetal fetuin and a 60Kd apoptosis-inducing protein have also been found in fetal serum and fresh embryo extract, respectively. Interestingly, a common characteristic of these embryonic apoptosis-inducing proteins is that they selectively induced apoptosis in cancer without affecting normal cells. For example, the 54 Kd protein selectively induced apoptosis in 10 out of 12 cancer cell lines without affecting 12 normal cell lines we tested. Fetal fetuin, on the other hand, selectively induced apoptosis in 5 cancer cell lines without affecting the 3 normal cell lines we tested. In vivo, tumor animal model study showed that fetal fetuin enhanced survival in leukemia-bearing mice and strongly inhibited the formation of prostate cancer in a PC-3 prostate cancer model in mice. A working hypothesis has been proposed to aid in the study of the mechanism by which the embryonic apoptosis-inducing proteins selectively induced apoptosis in cancer without affecting normal cells. This hypothesis states that due to the retro- differentiational characteristic of malignancy, cancer cells may re-express the signal transduction machinery for development-related apoptosis, which is otherwise to be normally expressed by embryonic, but not by adult cells. The embryonic apoptosis- inducing proteins may therefore induced apoptosis in cancer but not in normal cells and may be developed as an anticancer agent. This new concept may constitute a new approach for cancer therapy, which we tentatively designated as "Retro-differentiational Apoptosis Cancer Therapy". (R-ACT).*

Apoptosis (programmed cell death), in contrast to the cell death caused by cell injury (necrosis), is an active process of gene-directed cellular self-destruction and that it serves a biologically meaningful function (1). Apoptosis plays an important role in the human body from the early stages of embryonic development to the inevitable decline associated with old age. In the embryo, cell death is as essential as cell division and cell differentiation in properly regulating cell populations, organ formation and overall body shape. In normal adult tissue, apoptosis occurs continuously in slowly proliferating cell populations such as hepatic (2, 3) and adrenal cortical epithelium (4) and in rapidly proliferating populations such as intestinal crypt epithelium (5) and differentiating spermatogonia (6).

We have been particularly interested in the phenomenon that apoptosis is a highly active biological event in embryo. Glucksmann has enumerated 74 separated examples of embryonic cell death in 1950 (7). The factor(s) that induce(s) the enumerous apoptosis event in embryo is unclear. Inspired by the findings reported by Gerschenson's group that a heat and trypsin- sensitive, apoptosis-inducing factor was secreted by primary culture of rabbit endometrial cells (8), we hypothesize that certain embryonic tissues may secret soluble proteins that induce the highly active apoptotic event in embryo. To test this hypothesis, we tested whether apoptosis-inducing protein can be isolated from embryos. Evidences reported in the present study seem to support our hypothesis that embryo may secret certain apoptosis-inducing proteins.

Furthermore, based on the characteristics of these fetal apoptosis-inducing proteins and some well-known biological phenomena, we have proposed a novel approach for cancer therapy. In this paper, we will provide the evidence showing that the apoptosis-inducing proteins secreted by embryo may be developed as a novel class of anticancer agent. The scientific rationale behind this novel approach for cancer therapy will also be discussed.

Materials and Methods

Cell lines. The human promyelocyte leukemia cell line, HL-60 cells (CCL240; ATCC, Rockville, MD) were grown in RPMI 1640 (containing 20% fetal calf serum). The human metastatic prostate adenocarcinoma cell line, LNCaP (CRL 1740; ATCC) were grown in RPMI 1640 medium containing 15% FBS and Penicillin-Streptomycin (10,000 U/ml). The

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human colon carcinoma cell line, Colo 205 (CCL 222; ATCC) were grown in RPMI 1640 medium containing 10% FBS, sodium pyruvate, sodium bicarbonate and Penicillin-Streptomycin (10,000 U/ml). The human prostate adenocarcinoma cell line, PC-3 (CRL 1435; ATCC) were grown in Ham's F-12 medium containing 10% FBS and Penicillin-Streptomycin (10,000 U/ml). The human breast adenocarcinoma cell line, MCF-7 (HTB 22, ATCC) were grown in Eagle's MEM medium containing 10 % FBS, sodium pyruvate, non-essential amino acid, 1mM bovine insulin and Penicillin-Streptomycin (10,000 U/ml). The human lung carcinoma cell line, Calu-1 (HTB 54; ATCC) were grown in McCoy's 5A medium containing 10% FBS and Penicillin-Streptomycin (10,000 U/ml). Human normal lung fibroblast cell line, Wi-38 (CCL 75, ATCC) were cultured in Eagle's MEM medium containing 10% FBS, non-essential amino acid and Penicillin- Streptomycin (10,000 U/ml). The human normal lung cell line, CCD 39 Lu (CRL 1498, ATCC) were grown in Eagle's MEM medium containing 10 % FBS, Eagle's BSS, non-essential amino acid and Penicillin-Streptomycin (10,000 U/ml). The human normal colon fibroblast, CCD 18 Co (CRL 1459; ATCC) were grown in Eagle's MEM medium containing 10% FBS, Eagle's BSS, non-essential amino acid and Penicillin-Streptomycin (10,000 U/ml).

Reagents. Dulbecco's Modified Eagle Medium, McCoy's 5A medium and fetal bovine serum are purchased from GIBCO. The following media and reagents are purchased from Mediatech: Alpha Minium Essential Medium, Minimum Essential eagle Medium, Ham's F-12, RPMI- 1640, Ham's F-10, Non-essential amino acids, Penicillin-Streptomycin and Trypsin-EDTA.

Preparation of conditioned medium. C3H 10T1/2 cells were first grown in Alpha Modification of Eagle's Medium (alpha-MEM) containing 10 % fetal bovine serum (FBS) for 3 days. Cells were then washed with Phosphate Buffer saline (PBS) (3X100 ml) to remove serum and then grown in alpha-MEM containing no FBS for 4 days. The conditioned medium was then collected after cell debris was removed by filtration. Conditioned media of other cell lines are prepared by the same protocol in each individual suitable growth medium containing no FBS.

Apoptosis assays. Hoechst dye staining and MTS assays were used for apoptosis assay. The assays were performed in the presence of 5-10% FBS as described below:

(a) Hoechst dye assay. Cultured cells (5,000 cells) were seeded in 10 microliters growth medium containing 10%-20% Fetal bovine serum at 37 degree, 5% CO_2 in microtray plates (25 µl wells, Robbins Scientific Corp.). The tested sample (10 µl), after the buffer was changed to suitable growth medium, was added 12 hours after cells were seeded. After incubation of the tested sample with cells for 15 hours, two microliters of Hoechst 33258 (0.1 µg/ml in PBS) were added. Two hours later, cells that were stained with Hoechst dye were examined under a fluorescence microscope. The nuclei of apoptotic cells showing DNA condensation and fragmentation are easily identified by Hoechst dye staining. The percentage of apoptotic cells is calculated using the following equation:

$$\% \text{ Apoptotic cell} = \frac{\text{number of cell with DNA condensation and/or fragmentation}}{\text{Total cell number}}$$

(b) MTS assay: The MTS assay has been widely used as a cytotoxicity assay. The principle of this assay is that enzymes pressent in healthy, living cells may turn an oxidized dye into its reduced form which can be photometrically detected, whereas the unhealthy cells or dead cells lacking the enzymes are unable to turn the dye into the reduced form. This assay can therefore distinguish health cells from dead cells. The advantage and disadvantage of this assay is that it is quantitative but not qualitative. Any cell death, apoptotic or necrotic, may be detected by this method. This assay will be complementally used with the Hoechst dye assay in this study.

Preparation of genomic DNA for agarose gel analysis of DNA fragmentation. Cultured cells (10×10^6) were digested by incubating with digestion buffer (10 mM Tris-HCl, pH 8.0 containing 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate and 0.2 mg/ml proteinase K) at 50°C for 18 hr. Cell lysate was then extracted twice with phenol following by twice with phenol: chloroform (1:1) and twice with chloroform: isoamyl alcohol (24:1). Genomic DNA was extracted from the aqueous layer by cold 100% ethanol after proteins were removed by phenol and chloroform extraction. DNA pellet was rinsed for 5 minutes with 70% cold ethanol. Ethanol was decanted or aspired and pellet was dried completely. DNA pellet was then resuspended in TE buffer and digested with 0.2 mg/ml DNAase-free RNAase. Genomic DNA was developed by 2% agarose gel at 55 volts for 5 hr. Gel was stained by ethidium bromide (0.4 µg/ml) for at least 30 minutes.

Purification of apoptosis-inducing protein from the conditioned medium of 10T1/2 cells. HL-60 cells were routinely used for the purification of the protein inducing apoptosis in the conditioned medium of C3H10T1/2 cells. The purification procedure includes: DE 52 anion exchange chromatography, hydroxylapatite chromatography, heparin chromatography and G-75 sizing chromatography. This purification procedure leads to the purification and sequencing of a homogeneous protein with a molecular weight of 54 Kd on SDS-PAGE. The details of the purification and protein sequence will be reported somewhere else.

Purification of apoptosis-inducing protein from fresh mouse embryo. Twenty mouse embryos (5-15 days after gestation) were chopped into small pieces and homogenated in 50 ml PBS by a blender. Supernatant was collected by centrifugation and treated with one volume of methanol containing 0.05% trifluoroscetic acid. Precipitation was removed by filtration. Supernatant was then concentrated and buffer was exchanged to 10 mM Tris-HCl, pH 7.5 by repetitive concentration by a molecular sieve. The apoptosis-inducing activity was further purified by a procedure includes: Q2 anionic exchange chromatography, reverse phase chromatography and cationic exchange chromatography.

Purification of apoptosis-inducing protein from fetal bovine serum. LNCaP cells were routinely used in the assays for the purification of apoptosis-inducing protein present in fetal bovine serum. The apoptosis-inducing protein was purified by the following procedure: (1) Ammonium sulfate precipitation. (2) Hydroxylapatite treatment. (3) Heparin agarose treatment. (4) Reverse phase chromatography. The purity of the isolated apoptosis- inducing protein was determined by a SDS-polyacrylamide gel stained with silver staining. A single protein band with molecular weight of 65 Kd was obtained (Figure 2). This protein band was transfered to PVDF membrane and N-terminal amino acid sequence was determined.

Purification of fetuin from fetal bovine serum. Bovine fetuin was prepared by a modified Spiro method (9).

(1) Two hundred milliliters of 0.05 M zinc acetate containing 30% (V/V) ethanol was added to one hundred milliliters of fetal bovine serum (FBS), pH was adjusted to 6.4 by 1 M NH_4OH- NH_4Cl. Let stand for 15 hours at -5°C. (2) Collected the supernatant by centrifugation, added 1.0 M barium acetate and 95% ethanol to give 0.03 M barium acetate, 25% ethanol. Let stand for 2 hr at -5°C. (3) Collected the supernatant by centrifugation, added 95% ethanol to give 40% ethanol. Let stand for 16 hr at -10°C. (4) Collected the precipitate by centrifugation. Dissolved the pellet by phosphate buffer saline (PBS). Free zinc acetate and barium acetate were removed by repetitive concentration through a molecular seive against 20 volumes of PBS for 4 times. The modified Spiro method distincts from the original Spiro method in that not extensive dialysis nor chelating agent (Trisodium Citrate) treatment were used in our preparation. The purified fetuin showed a single protein band with apparent molecular weight of 65 Kd on SDS-PAGE.

Preparation of Zn-charged fetal fetuin. The fetal fetuin isolated from fetal bovine serum by the modified Spiro method was incubated with high

Table I. *Apoptosis-inducing activity of the conditioned media of various cell lines.*

Conditioned medium		Cell line tested (% apoptosis)				
		HL-60	A172	LNCaP	MDA	Hep3B
C3H 10T1/2	(Mousewhole embryo)	92%	1%	87%	2%	1%
F-9	(Mouse embryonic carcinoma)	85%	82%	2%	1%	38%
SVT2	(Mouse embryo)	87%	76%	3%	2%	55%
FHS	(Human whole embryo)	85%	1%	1%	2%	34%
WSI	(Human fetal skin)	60%	32%	28%	9%	35%
CCD 112 CoN	(Humanfetal colon fibroblast)	2%	1%	2%	1%	1%
NIH 3T3	(Mouse embryo)	4%	1%	4%	2%	5%
Cos-1	(Adult monkey kidney)	2%	1%	3%	2%	4%
BUD-8	(Adult human skin)	4%	2%	5%	3%	2%
BT-549	(Adult human breast)	2%	3%	7%	2%	2%
A431	(Adult human skin)	3%	2%	7%	2%	2%
NRK-49T	(Adult rat kidney)	2%	1%	6%	3%	2%
WEHI-274.1	(Adult mouse monocyte)	5%	2%	7%	3%	2%
PC-12	(Adult rat pheochromocytoma)	3%	2%	5%	3%	2%

concentration of zinc acetate (0.25M) at room temperature for 1 hr. The unbound metal ion was then removed by repetitive concentration by a molecular sieve against 20 volumes of PBS for 4 times.

Preparation of mature fetuin. The mature fetuin was prepared by the same method for fetal fetuin preparation, except that adult bovine serum, instead of fetal serum, was used for the preparation.

In vivo, tumor animal study (P388 leukemia model). Forty DBA/2 female mice (17-20 grams) kept on a standard diet and water ad libitum were innoculated with tumor cell line P388D1 (ATCC; CCL46). Freshly harvested tumor cells were diluted with saline. One million cells were injected intraperitoneally. The mice were randomly segregated into groups of 10 and housed in shoe box cages. Zn-charged fetal fetuin was intraperitoneal injected into group I (1 mg/Kg), group II (10 mg/Kg) and Group III (100 mg/Kg). A control group (group IV) was included and injected with saline on the days that the tested animals were treated. The injection were continued for 10 days. Mortalities were recorded daily. Results are expressed as the percentage increase in life span(ILS):

$$ILS = \frac{100 \times \text{Median Life Span Treated} - \text{Median Life Span Controlled}}{\text{Life Span Controlled}}$$

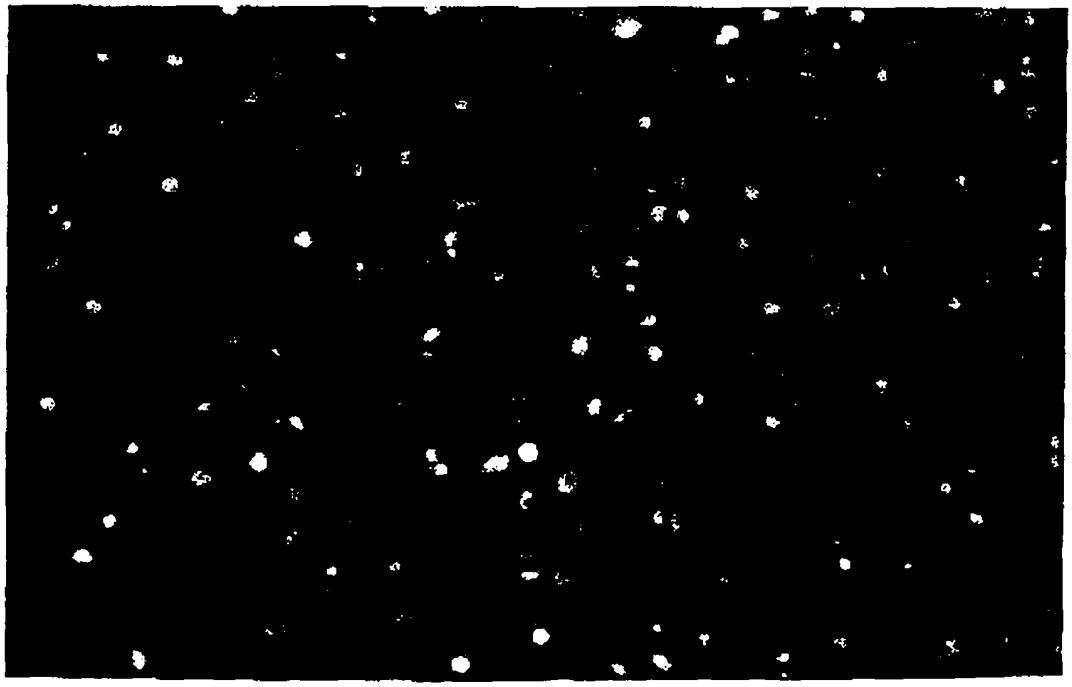
In vivo, tumor animal study (PC-3 prostate cancer model). Male nude mice (25-30 g) were used in this study. The prostate adenocarcinoma cell; PC-3 (2 million cells) were injected on the upper half of the dorsal thorax of the mice. The tumors were allowed to grow for 6 weeks. The mice in the control group (no treatment) received 0.1 ml saline intraperitoneally for 5 days and the mice in the treated group received 50 mg/Kg fetuin. Treatment started the day after tumor inoculation for 5 days. Six weeks after the inoculation of tumor, mice were sacrificed and tumors were weighted.

Results

(1) The conditioned media of embryonic cell lines contain apoptosis-inducing activity. To test the hypothesis that embryo may secret apoptosis-inducing proteins, we tested whether apoptosis-inducing activity can be detected in the conditioned medium of cell line derived from embryo. The conditioned medium prepared as described in Materials and Methods was concentrated 20 fold and buffer was exchanged to the same growth medium as that of the individual tested cell line by a molecule sieve. The tested cell line was incubated with the concentrated conditioned medium for 15 hr. The apoptosis assay were performed by Hoechst dye staining as described. The apoptotic cells were identified by DNA condensation and/or DNA fragmentation, which was demonstrated by a more intense fluorescent light of the nucleus and the breakage of nucleus, respectively. As shown in Table I, among the seven cell lines derived from embryo (cell line1-7), the conditioned media of five embryonic cell lines (C3H10T1/2, F-9, SVT2, FHS and WSI) contain activity causing DNA condensation and fragmentation. None of the conditioned media of the seven cell lines derived from adult tissues contains the same activity.

(2) The extract of fresh mouse embryo also contains apoptosis-inducing activity. To further test the notion that embryo may secret apoptosis-inducing protein, we tested whether apoptosis-inducing activity can be detected in fresh embryo

HL - 60, Control



HL - 60 + Fresh mouse emdryo extract

Figure 1. *Apoptosis induced in HL 60 cells by the apoptosis-inducing protein isolated from fresh mouse embryo. The apoptosis-inducing protein (300 µg/ml) isolated from mouse embryo (15-15 days after gestation. PelFr(2) was incubated with HL-60 cells for 15 hr. The assayed medium both control and experimental) contains 50% growth medium, 50% PBS and 10% FBS. Cells were stained with Hoechst dye 33258 (0.1 µg/ml) for 2 hr and inspected under a fluorescence microscope.*

extract. The supernatant of methanol-treated embryo homogenate was found to contain an activity inducing apoptosis in HL-60 cells. This activity has been partially purified by a procedure described as in Materials and Methods. A protein with apparent molecular weight of 60 Kd has been obtained. As shown in Figure 1, this protein induced DNA condensation and fragmentation in HL-60 cells. However, LNCaP and MCF-7 cells were not affected by this protein, suggesting the selectivity of inducing apoptosis of this protein (data not shown).

(3) The fetal bovine serum also contains apoptosis-inducing activity. We further tested whether fetal serum also contains apoptosis-inducing activity. When fetal bovine serum was first fractionated by treating with 60% saturated ammonium sulfate, an apoptosis inducing activity was found in the resolved pellet of the ammonium sulfate treatment. Using HL-60 and LNCaP cells, this activity was purified to homogeneity by a procedure as described in Materials and Methods. As shown in Figure 2, a protein with molecular weight of 65 Kd was obtained. The N-terminal amino acid sequence of this protein (I-P-L-D-P-V-A-G-Y-K) reveals that it is a protein called "Fetuin"--a fetal protein functions to control embryogenesis.

(4) Characterization of the apoptosis-inducing activity secreted by embryo. The above results showing that apoptosis-inducing activity can be found in embryonic cell lines, fresh embryo extract and fetal serum suggest that embryo may secret apoptosis-inducing factors. For the purpose of characterizing the apoptosis-inducing factor secreted by embryo, we focus on the study of the apoptosis-inducing factor secreted by C3H 10T1/2 cell line and fetal fetuin found in fetal serum based on the following reasons: (1) the conditioned medium of C3H 10T1/2 cells contains highest apoptosis-inducing activity. (2) the identification of fetuin as the apoptosis-inducing protein present in fetal serum enables us to study embryonic apoptosis-inducing protein on the molecular level. Furthermore, the availability of fetuin (a abundant fetal protein) enables us to study this protein on cellular level and animal level as well.

(4.1) Characterization of the poptosis-inducing activity secreted by C3H 10T1/2 cells: a 54 Kd protein selectively induced apoptosis in cancer without affecting normal cells. To characterize the conditioned medium of C3H 10T1/2 cells, HL-60 cells grown in 100 µl RPMI 1640 containing 20% fetal calf serum were incubated with 50 µl of 20 fold concentrated conditioned medium (buffer was exchanged to RPMI) for 15 hr. The characteristic of apoptosis was observed both morphologically and biochemically in up to 80% of the HL-60 cells. Figure 3 shows the biochemical characteristic of HL-60 cell undergoing apoptosis induced by the conditioned medium of C3H 10T1/2 cells. In Figure 3(A), genomic DNA was extracted from the HL-60 cells incubated with control medium (20 fold concentrated non-conditioned medium that has been exchanged to RPMI medium). In Figure 3(B), genomic DNA was extracted from the HL-60 cells incubated with conditioned medium (20 fold concentrated, exchanged to RPMI medium) for 15 hr. A so-called "DNA ladder" was observed, suggesting the induction of apoptosis on HL-60 cells by the conditioned medium. The apoptosis-inducing activity found in the conditioned medium of C3H 10T1/2 cells is heat and proteinase K sensitive, suggesting the protein nature of this activity (data not shown). HL-60 cells were routinely used for the purification of the protein inducing apoptosis in the conditioned medium of C3H 10T1/2 cells. As shown in Figure 4, an apoptosis-inducing protein with a molecular weight of 54 Kd on SDS-PAGE has been obtained. The N-terminal sequence (19 amino acids) of this protein has been determined and was found not to match any known protein on BLAST serach. The amino acid sequence of this protein will be reported elsewhere. To further characterize the 54 Kd protein, various cell lines were incubated with a control medium or the purified 54 Kd protein (250 µg/ml) for 15 hr and then stained with Hoechst dye for 2 hr. As shown in Figure 5, the nuclei of the cells that have been incubated with the control medium were normal and intact (Figure 5). However, the nuclei of the HL-60, LNCaP and JEG-3 cells that have been incubated with the conditioned medium showed the characteristics of apoptosis. First, the conditioned medium caused the condensation of nucleus, which was demonstrated by the more intense fluorescent light compared with the control nucleus in Figure 5. Secondly, the nuclear condensation was accompanied by the fragmentation of DNA, which was demonstrated by the breakage of nucleus as shown. Strikingly, while the 54 Kd protein strongly induced apoptosis on the three cancer cell lines: HL-60 (human promyclocyte leukemia), LNCaP (human metastatic prostate adenocarcinoma) and JEG-3 (human choriocarcinoma), the three normal cell lines: NSF-5 (Factor dependent hemopoietic cells), CCD 39 Lu (human normal lung fibroblast) and CCD 18 Co (human normal colon) were not affected by the 54 Kd protein (Figure 5). The apoptosis-inducing selectivity of the 54 Kd protein was then systematically tested on 12 cancer cell lines and 12 normal cell lines using the Hoechst dye staining and MTS assays. We found that among the 12 cancer cell lines tested, LNCaP (human metastatic prostate adenocarcinoma), HL-60 (human promyelocyte leukemia), G-401 (Wilm's tumor), Hep G2 (hepatocarcinoma), T-84 (colon carcinoma), JAR (human choriocarcinoma) and JEG-3 (human choriocarcinoma) are highly sensitive (>90% apoptosis) to this protein. Whereas, HS-294T (melanoma), epitheloid carcinoma (HeLa) and Hep 3B (hepatocarcinoma) are moderately (20- 40% apoptosis) affected by this protein. A172 (glioblastoma) and CaLu-1 (lung carcinoma) are not affected by this protein. On the other hand, none of the 12 normal cell lines we tested is affected by this protein. These normal cell lines include: lung fibroblast, normal prostate, foreskin fibroblast, bone marrow,

Figure 2. *SDS-polyacrylamide gel electrophoresis of purified apoptosis-inducing protein from fetal bovine serum. The apoptosis-inducing protein was purified by the procedure as described in Materials and Methods from fetal bovine serum. protein sample (20 ng in 20 µl) was dissolved by 4-20% gradient gel and silver stained. In a separated run, about 200 ng of protein sample was run on gel and stained with Coomassie blue. The protein band was then transfered to a PVDF membrane and N-terminal amino acid sequence was determined. An amino acid sequence of I-P-L-D-P-V-A-G-Y-K was obtained.*

Figure 3. *Agarose gel (2%) electrophoresis of DNA extracted from cultures of HL 60 cells incubated with the conditioned medium of C3H 10T1/2 cells. HL-60 cells (10^6 cells) grown in petri dish (100X 15 mm) were incubated with (A) control buffer (RPMI containing 10% FBS) or (B) the conditioned medium (MEM. x 20, exchanged to RPMI. containing 10% FBS) for 15 hr. Cells were then collected and digested by incubating with digestion buffer 10 mM Tris-HCl, pH 8.0 containing 10% mM NaCl. 25 mM EDTA. 0.5% sodium dodecyl sulfate and 0.2 mg/ml proteinase K) at 50°C for 18 hr. Cell lysate was then extracted twice with phenol followed by twice with phenol: chloroform (1:1) and twice with chloroform: isoamyl alcohol (24:1). Genomic DNA was extracted from the aqueous layer by cold 100% ethanol after proteins were removed by phenol and choroform extraction. DNA pellet was rinsed for 5 minute with 70 % cold ethanol. Ethanol was aspired and pellet was dried completely. DNA pellet was then resuspended in TE buffer and digested with 0.2 mg/ml of DNAase- free RNAase. Genomic DNA was developed by 2% agarose gel at 55 volts for 5 hr. Gel was stained by ethidium bromide (0.4 µg/ml) for at least 30 minutes.*

Figure 4. *SDS-polyacrylamide gel electrophoresis of purified apoptosis-inducing protein from the conditioned media of C3H 10T1/2 The apoptosis-inducing protein was purified by the procedures as described in Materials and Methods from the conditioned medium. Protein sample (20 µl) was dissolved by 4% gradient gel and silver stained. In a separated run, about 200 ng of protein sample was run on gel and stained with Coomassie blue. The protein band was then transfered to a PVDF membrane and N-terminal amino acid sequence was determined.*

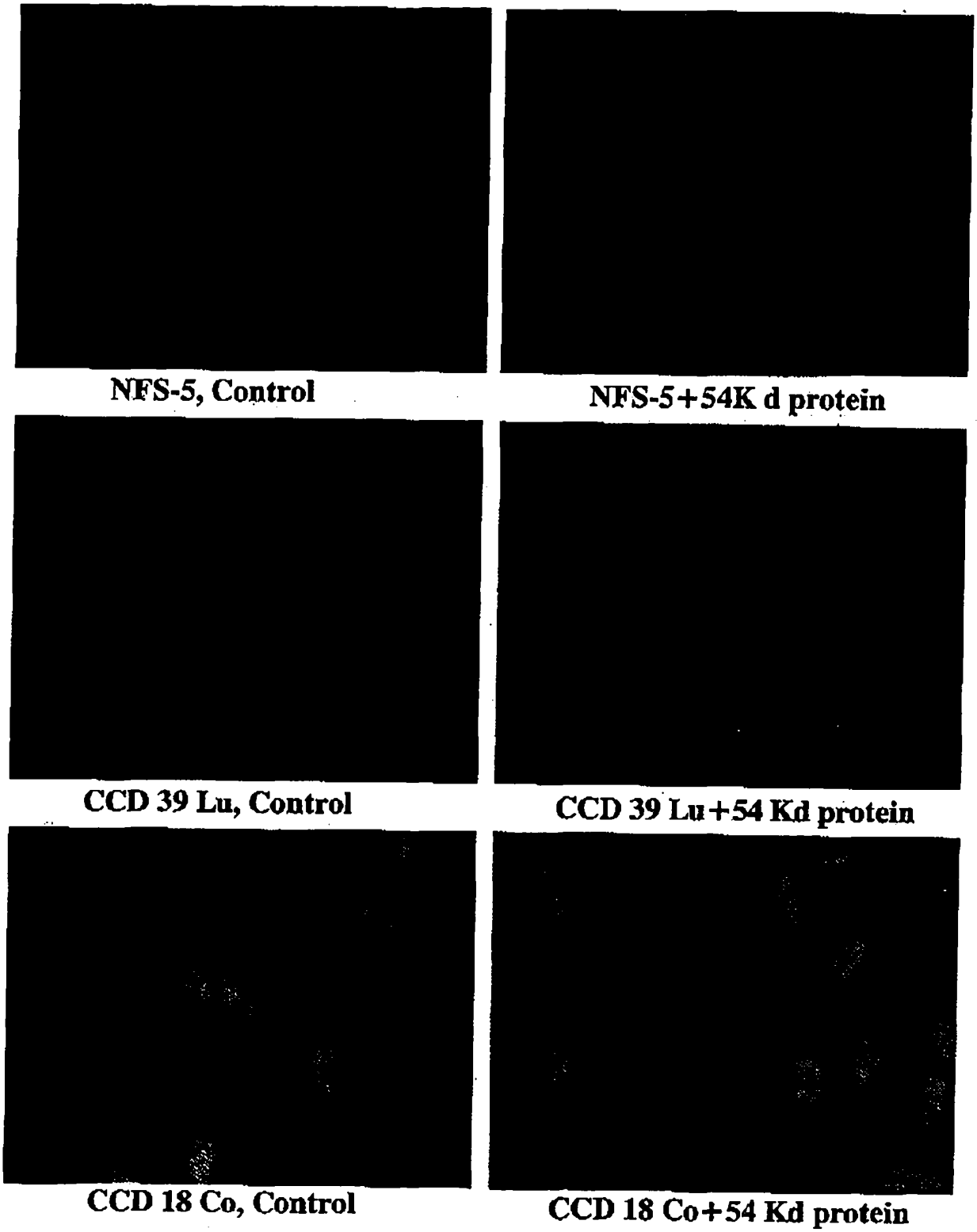


Figure 5.

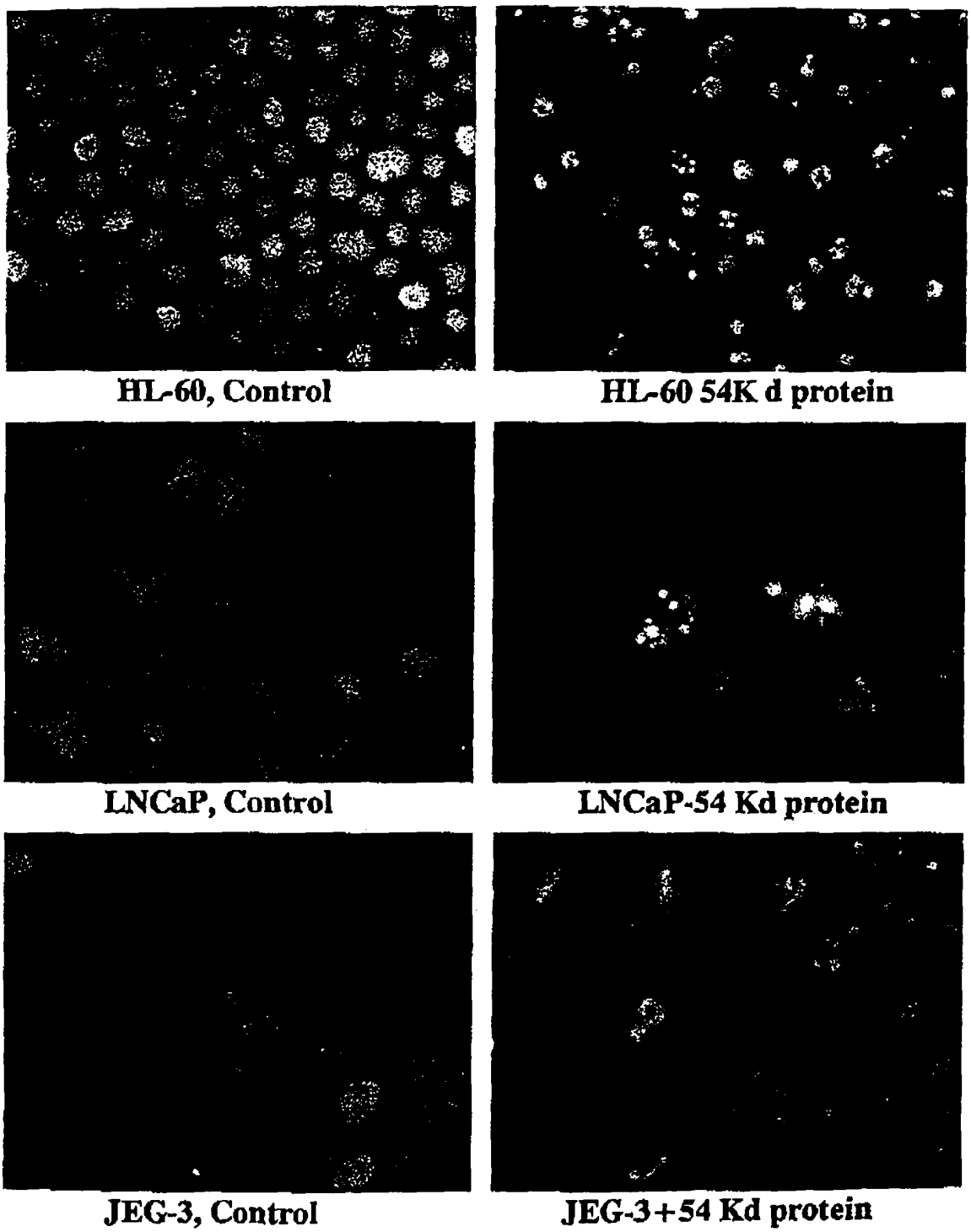


Figure 5. The 54 Kd protein induced apoptosis on cancer cell lines without affecting normal cell lines The following cell lines were incubated with the 54Kd protein isolated from the conditioned medium of C3H 10T1/2 cells (250 µg/mL dissolved in PBS) for 15 hr. The assayed medium (both control and experimental) contains 50% growth medium, 50% PBS and 7-10% FBS. Cells were stained with Hoechst dye 33258 (0.1 ug/ml) for 2 hr and inspected under a fluorescence microscope. Cells with characteristics of DNA condensation or fragmentation were quantified as described in Materials and Methods. Cancer cell lines: HL-60 (leukemia), LNCaP (prostate cancer) and JEG-3 (choriocarcinoma) Normal cell lines: NFS-5 (hemopoietic progenitor cells) CCD39 Lu (lung fibroblast) and CCD 18 Co (Colon).

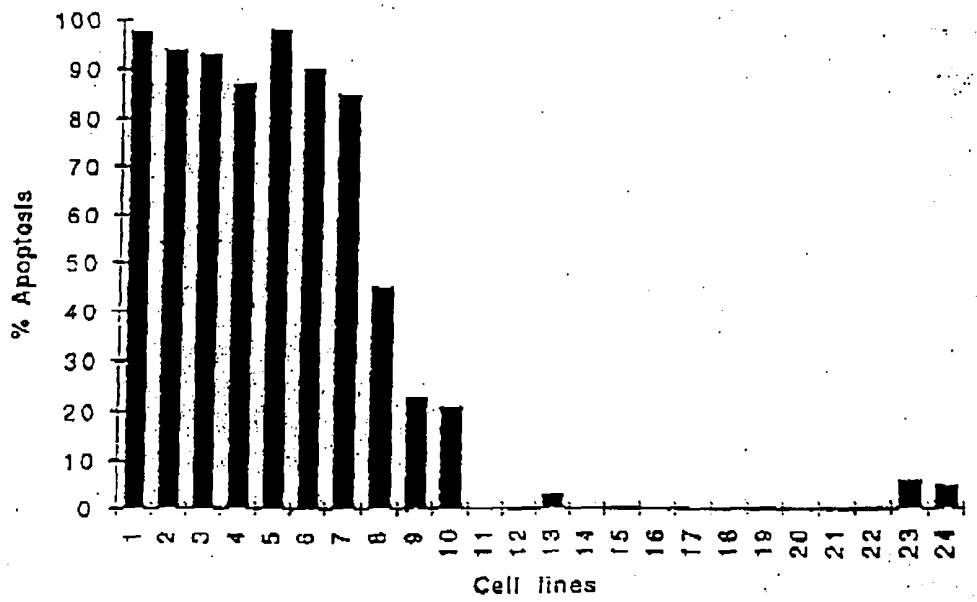


Figure 6. *Induction of apoptosis on tumor (1-12) and normal (13-24) cell lines by the 54 Kd protein isolated from conditioned medium of C3H 10T1/2. The following cell lines were incubated with the 54Kd protein isolated from the conditioned medium of C3H 10T1/2 cells (250 μg/mL dissolved in PBS) for 15 hr. The assayed medium (both control and experimental) contains 50% growth medium, 50% PBS and 7-10% FBS. Cells were stained with Hoechst dye 33258 (0.1 μg/ml) for 2 hr and inspected under a fluorescence microscope. Cells with characteristics of DNA condensation or fragmentation were quantified as described in Materials and Methods. Tumor cell lines: 1. Choriocarcinoma (JAR) 2. Leukemia (HL-60). 3. Wilm's tumor (G-401). 4. Choriocarcinoma (JEG3). 5. Prostate cancer (LNCAP). 6. Hepatocarcinoma (Hep G2).7. Colon carcinoma (T84). 8. Melanoma (HS- 294T). 9. Epithelioid carcinoma (HeLa).10. Hepatocarcinoma (Hep 3B). 11. Glioblastoma (A 172). 12. Lung carcinoma (CaLu- 1). Normal cell lines: 13. Human lung fibroblast (CCD 39 LU). 14. Human lung (WI-38). 15. Human foreskin fibroblast (HS-27). 16. Human foreskin fibroblast (HS-68). 17. Rat vascular smooth muscle (SM). 18. Rat bone marrow (MB). 19. Hemopoietic progenitor cells (32 DC3). 20. Hemopoietic progenitor cells (32 DC23). 21. Pre-B cell leukemia, factor dependent (NFS- 5). 22. Normal prostate (E1.4). 23. Factor dependent hemopoietic cells SLD).24. Factor dependent hemopoietic cell (SRT 003).*

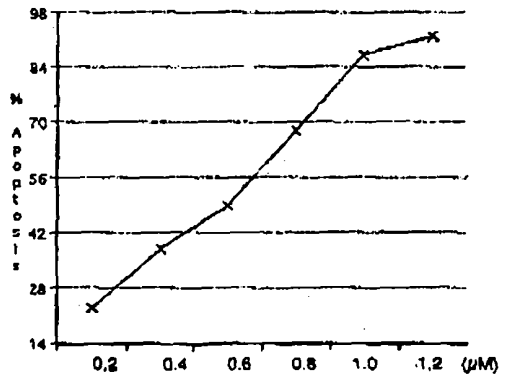


Figure 7. *The 54 Kd protein induced apoptosis in a dose-dependent manner. The 54 kd protein was purified as described in Materials and Methods. After the buffer was changed to PBS by repetitive concentration by a molecule sieve, the 54 Kd protein (2 μM) was incubated with LNCaP cells for 15 hr. Cells were then stained by Hoechst 33258 (0.1 μg/ml) for 2 hr and inspected under a fluorescence microscope. Each data represents the average of three assays.*

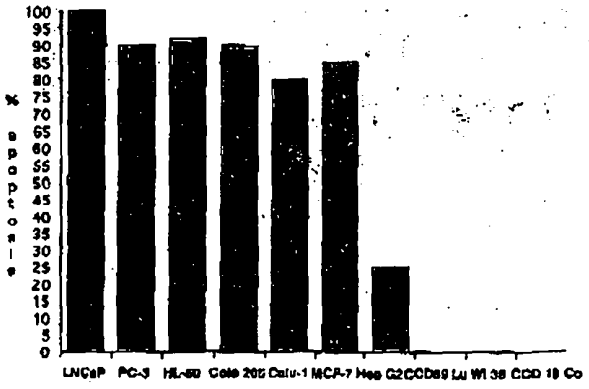
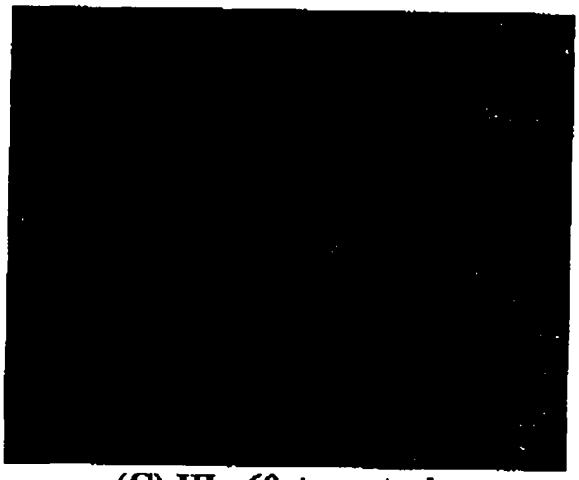


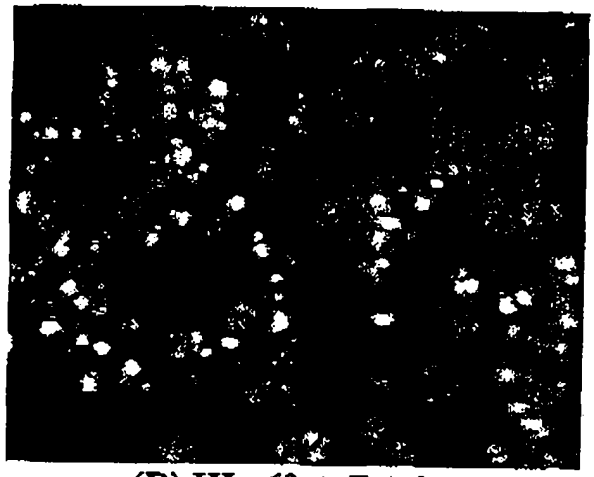
Figure 8. *Fetuin induced apoptosis in various cancer cell lines. Fetuin was purified by a modified Spiro method as described in Materials and Methods. After the buffer was changed to PBS by repetitive concentration* by a molecule sieve, fetuin (250 μg/ml) was incubated with cultured cells for 10 hr. Cells were then stained by Hoechst 33258 (0.1 μg/ml) for 2 hr and inspected under a fluorescence microscope. Each data represents the average of three assays. * Trace amount of Zn and Ba may remain in the fetuin preparation. All the cell lines are not affected by Zn or Ba alone at all the concentrations tested (up to 66 μM).*

(A) LNCaP + control

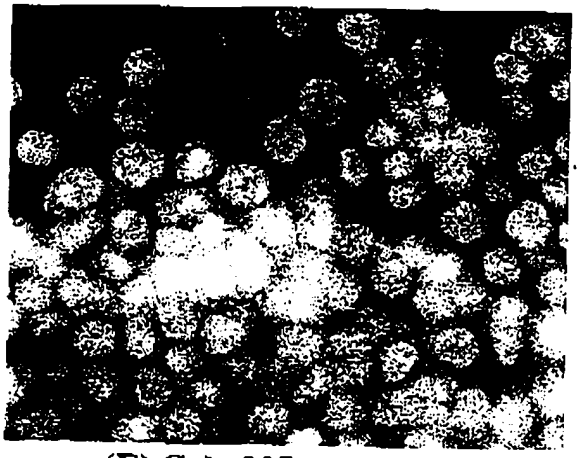
(B) LNCaP + Fetuin



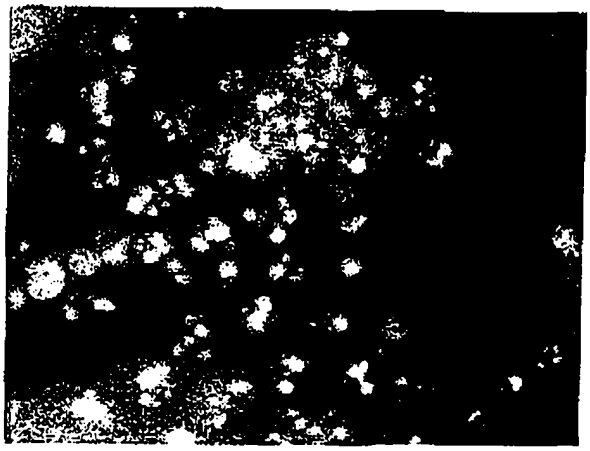
(C) HL -60 + control



(D) HL -60 + Fetuin



(E) Colo 205 + Control



(F) Colo 205 + Fetuin

Figure 9. *A,B,C,D,E,F.*

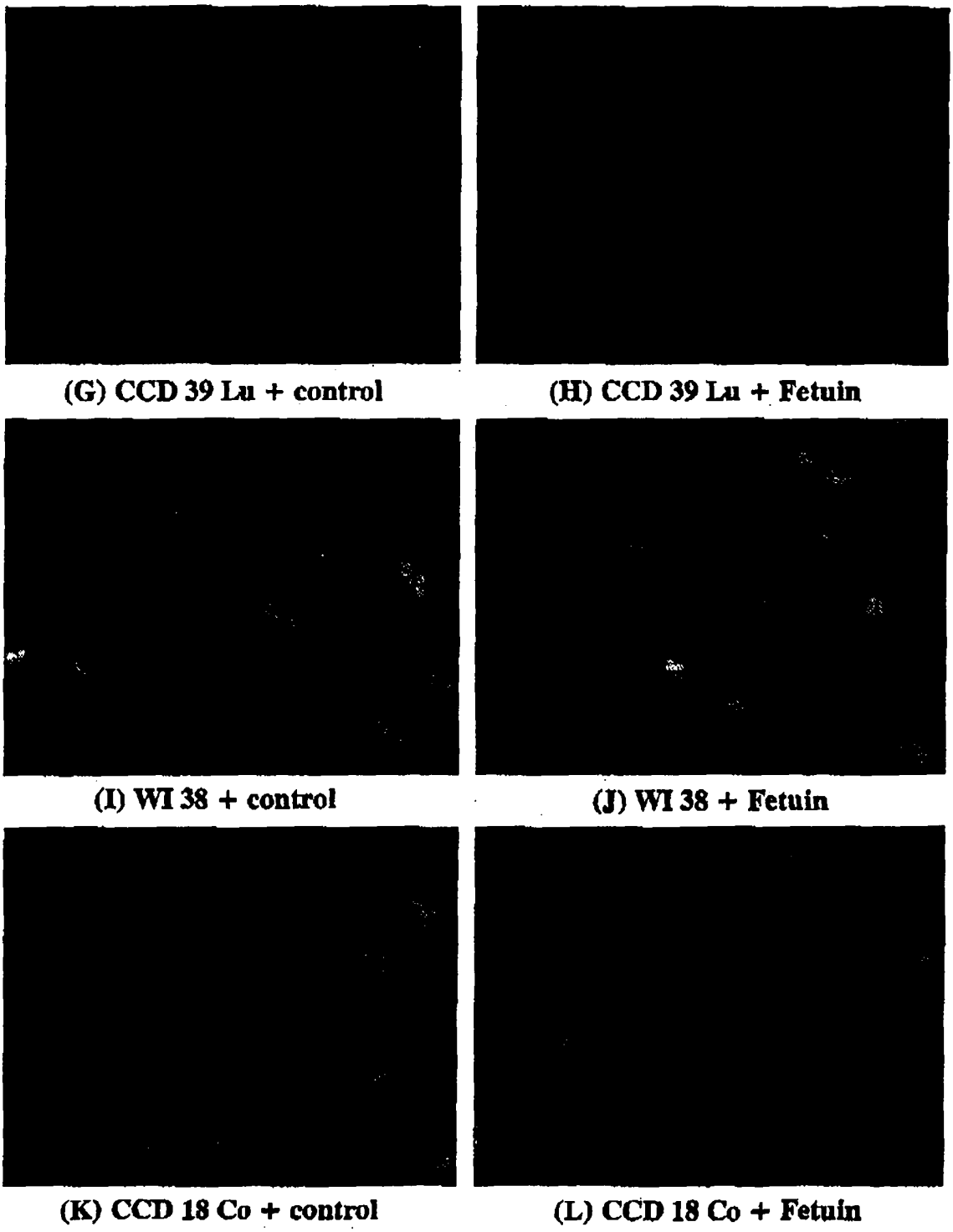


Figure 9. *The effect of fetuin on various cell lines. The fetuin (2 μM) purified by a modified Spiro method was incubated with the following cell lines: LNCaP (A and B), HL-60 (C and D), Colo 205 (E and F), CCD 39 Lu (G and H), CCD 18 Co (I and J) and WI 38 (K and L) for 15 hr. Cells were then stained by Hoechst 33258 (0.1 μg/ml) for 2 hr and inspected under a fluorescence microscope.*

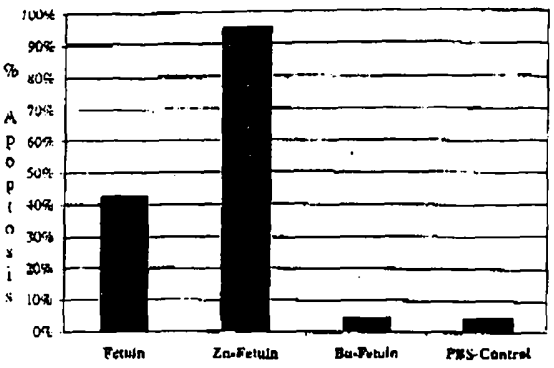


Figure 10. *Induction of apoptosis in LNCaP cells by fetuin, Zn-charged fetuin and Ba-charged fetuin. The fetuin (5 μM) purified by a modified Spiro method was incubated with zinc acetate or barium acetate (0.25 M) at room temperature for 1 hr. Free ions were removed by repetitive concentration against 20 volumes of PBS three times*. Fetuin, Zn-charged fetuin, Ba-charged fetuin and PBS were then separately incubated with LNCaP cells for 4 hr. Percentage of cells under apoptosis was determined by Hoechst dye staining and confirmed by MTS assay. Each data represents the average of two assays. * Zn-charged or Ba-charged fetuin may contain free Zn or Ba. All the cell lines are not affected by Zn or Ba alone at all the concentrations tested (up to 66 μM).*

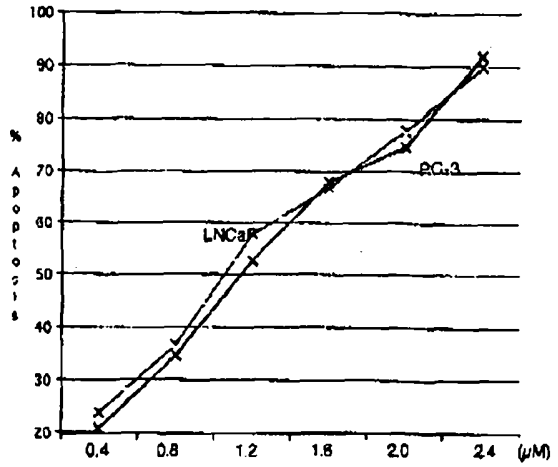


Figure 11. *Fetuin induced apoptosis in a dose-dependent manner. Fetuin was purified by a modified Spiro method as described in Materials and Methods. After the buffer was changed to PBS by repetitive concentration by a molecule sieve, fetuin (2 μM) was incubated with LNCaP cells for 6 hr. Cells were then stained by Hoechst 33258 (0.1 μg/ml) for 2 hr and inspected under a fluorescence microscope. Each data represents the average of three assays.*

hemopoietic progenitor cells and primary vascular smooth muscle cells (Figure 6). This result suggests that the 54 Kd protein isolated from the conditioned medium of C3H 10T1/2 cells selectively induced apoptosis in cancer without affecting normal cell lines. The 54 Kd protein was found to induce apoptosis in a dose-dependent manner. As shown in Figure 7, various concentrations of the 54 Kd protein were incubated with LNCaP cells for 15 hr. The 50% lethal dose (LD50) was determined to be 0.6 μM.

(4.2) Characterization of the apoptosis-inducing activity in fetal bovine serum: identification of fetal fetuin as a protein selectively induced apoptosis in cancer without affecting normal cells. As shown in Figure 2, the apoptosis-inducing protein isolated from fetal bovine serum has a N-terminal amino acid sequence of (I-P-L-D-P-V-A-G-Y-K), which by GenBank BLAST search, is identified to be that of fetal bovine fetuin. To further confirm that bovine fetuin *per se* contains apoptosis-inducing activity, we tested the fetuin purified from fetal bovine serum by a modified Spiro method. As shown in Figure 8, the fetuin purified by a modified Spiro method strongly induced apoptosis in certain cancer cell lines such as: LNCaP (human metastatic prostate adenocarcinoma), PC-3 (human prostate adenocarcinoma), HL-60 (human promyelocyte leukemia), MCF-7 (human breast adenocarcinoma), Colo 205 (human colon carcinoma) and Calu-1 (human lung carcinoma). Non-cancerous, normal cell lines

Table II. *LD 50 of fetuin-induced apoptosis in various cell lines.*

Cell lines	LD 50
LNCaP	1 μM
PC-3	1 μM
Colo 205	5 μM
Calu-1	4 μM
HL-60	5 μM
MCF-7	8 μM
Hep G2	20 μM
CCD 39LU	> 100 μM
CCD 18Co	> 100 μM
WI 38	> 100 μM

Various concentration of Zn-charged fetuin prepared as described in Fig. 8 were incubated with cell lines for 6 hr. The assay condition was as described in Fig. 8. % of cell under apoptosis was determined was determined by Hoechst dye staining and confirmed by MTS assay. The concentration of Zn-charged fetuin for the induction of 50% of cells under apoptosis (LD 50) was determined. Each data represents the average of three assays.

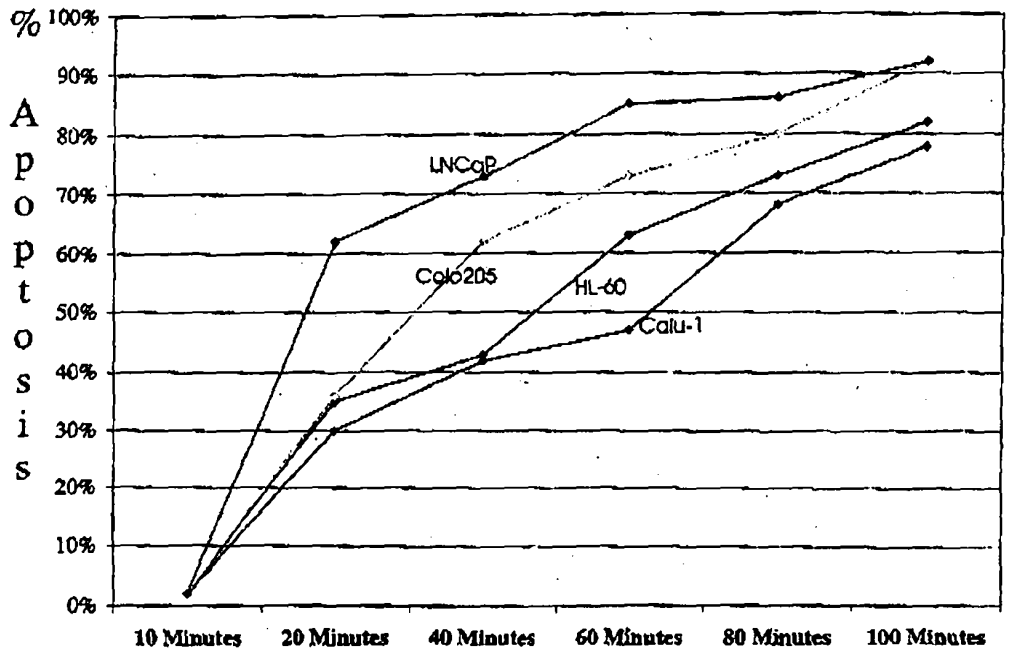


Figure 12. *Time course of the induction of apoptosis in cancer cell lines. Zinc-charged fetuin (5 μM) prepared as described in Figure 3 was incubated with cell lines. At various time interval, cells under apoptosis (cell shrinkage and DNA condensation) were determined by Hoechst dye staining and inspected under a fluorescence microscope. Each data represents the average of two assays.*

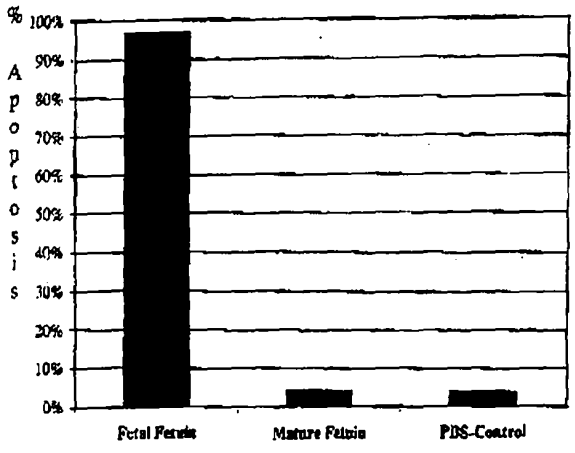


Figure 13. *Fetal, but not mature fetuin contains apoptosis-inducing activity. Fetuins were separately purified from fetal bovine serum or mature bovine serum by a modified Spiro method as described. Equal amount of fetal or mature fetuin (5 μM) was incubated with LNCaP cells for 15 hr. Percentage of cells under apoptosis was determined by Hoechst dye staining and confirmed by MTS assay. Each data represents the average of two assays.*

Table III. *Effect of fetal fetuin in P388 leukemia animal model. Forty DBA/2 female mice (17-20 grams; Simonsen Laboratories, Inc., Gilroy, CA) kept on a standard diet and water ad libitum were innoculated with tumor cell line P388D 1 (ATCC CCL46). The mice were randomly segregated into groups of 10. Zn-charged fetal fetuin (10mg/ml) prepared as described in Materials and methods were intraperitoneal injected into group I (0.002 mL/mouse, make up to 0.5 ml), group III (0.02 mL/mouse, make up to 0.5 ml) and group III (0.2 mL/mouse, make up to 0.5 ml). A control group (group IV) was injected with 0.5 ml saline on the days that the tested animals were treated. The injections were continued for 10 days. Mortalities were recorded daily for 60 days. Results are expressed as the percentage increase in life span (ILS):*

$$ILS = \frac{100 \times \text{Median Life Span Treated} - \text{Median Life Span Controlled}}{\text{Life Span Controlled}}$$

Group	No. Mice	Dose	Survivors (day)	(ILS) Increased Life Span
I	10	0.002 ml	1 (31)	29 %
II	10	0.02 ml	1 (29)	17.2 %
III	10	0.2 ml	8 (58)	141 %
IV	10	0.5 ml saline	0 (24)	----

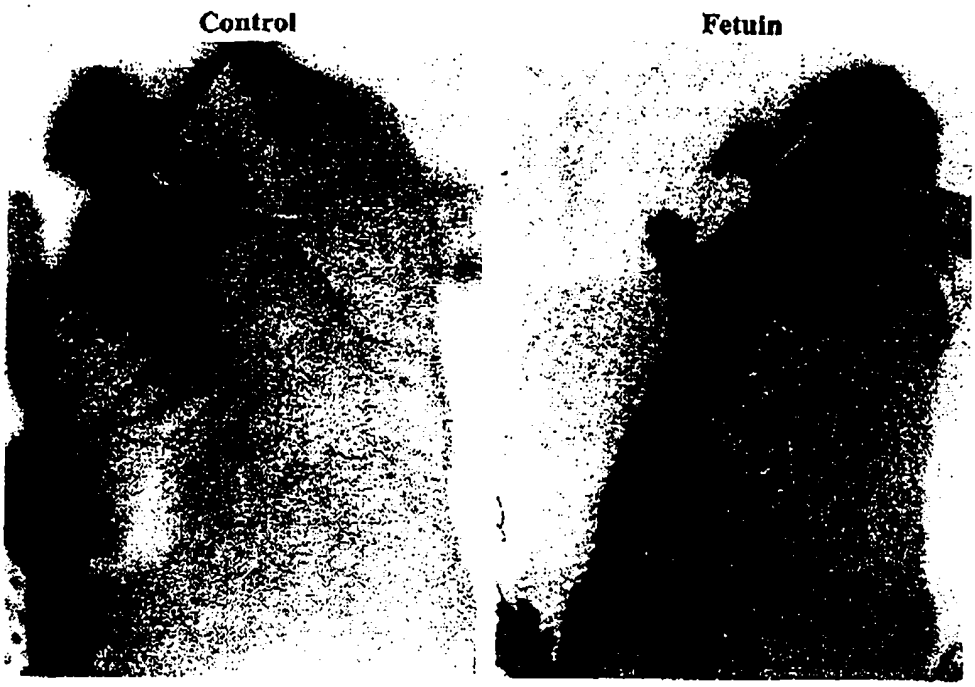


Figure 14. *Fetuin completely inhibited prostate cancer formation in PC-3 prostate cancer model in mice. Male nude mice (25-30 g) were inoculated with PC-3 cells as described in Materials and Methods. Six weeks after the inoculation of tumor, nude mice were sacrificed and tumors were weighted. While tumors (average weight=325 mg) developed in 13 of 13 mice in the control group (treated with saline), none of the 9 mice treated with fetuin (50 mg/Kg) developed any tumor.*

such as: WI-38 (human normal lung fibroblast), CCD 39 Lu (human normal lung) and CCD 18 Co (human normal colon fibroblast), on the other hand, are not affected by fetuin. The morphological demonstration of the induction of apoptosis in cancer cell lines by fetuin is shown in Figure 9. The incubation of fetuin (2 μM) results in the condensation and fragmentation of DNA, which are demonstrated by a more intense fluorescence light and breakage of nuclei, respectively (Figure 9B, 9D and 9F). Normal cell line, Wi-38, CCD 39 Lu and CCD 18 Co, as shown in Figure 9G-Figure 9L, are not affected.

(4.3) Zinc is an important factor for the apoptosis-inducing activity of fetuin. The fetuin isolated by the modified Spiro method, which involved zinc precipitation followed by barium treatment was incubated with high concentration of zinc acetate (0.25 M) or barium acetate (0.25 M) at room temperature for 1 hr, The unbound metal ion was then removed by repetitive concentration by a molecular sieve against 20 volumes of PBS for 4 times. The Zn- charged or Ba-charged fetuin were then tested in LNCaP cells. As shown in Figure 10, Zn-charged fetuin strongly and rapidly induced apoptosis with an activity higher than that of the originally uncharged fetuin. The induction of apoptosis in LNCaP cells by Zn-charged fetuin is so strong and rapid that almost 100% of LNCaP cells were under apoptosis in 4 hr. On the other hand, Ba-charged fetuin failed to show any activity inducing apoptosis in LNCaP cells. The same result is obtained in an assay using Colo 205 cells. This result suggests that Zn is necessary for fetuin to induce apoptosis. Substitution of Zn by barium completely abolished the apoptosis- inducing activity of fetuin. As shown in Figure 11, Zn-charged fetuin induced apoptosis in various cancer cell lines in a dose-dependent manner. The LD 50 of fetuin-induced apoptosis in each cell line is shown in Table II. the relative sensitivity of cancer cell lines to fetuin is: LNCaP=PC-3>Calu-1>HL-60>Colo205>MCF-7>HepG2, whereas the three normal cell lines: CCD 39 Lu, CCD 18 Co and Wi-38 seem to be insensitive to Zn-charged fetuin. Figure 12 showed the time course of the induction of apoptosis in tumor cells by Zn-charged fetuin. We found that Zinc- fetuin rapidly induced apoptosis in cancer cell lines.

(4.4) Fetal, but not mature fetuin contains apoptosis-inducing activity. fetal fetuin and mature fetuin isolated from fetal bovine serum and mature bovine serum respectively, were tested on LNCaP cells. We found that the fetuin isolated

from fetal bovine serum strongly induced apoptosis in LNCaP cells, whereas, the fetuin (at the same protein concentration) isolated from mature bovine serum showed no activity inducing apoptosis (Figure 13). This result may suggest the molecular change of fetuin during development.

(4.5) Anti-leuhemia effect of fetal fetuin in mice. Table III showed the increase of survival of leukemia-bearing mice treated with fetal fetuin. It was found that while 100% of untreated mice (injected with saline) were dead after 24 days, 80% (8 out of 10) of mice injected with high dose (100 mg/Kg) of fetal fetuin survived after 58 days. This experiment showed that the treatment of fetal fetuin increased the life span of p388 leukemia-bearing mice by 141%.

(4.6) Fetuin completely inhibited prostate cancer in mice. Male nude mice (25-30 g) were used in this study. The prostate adenocarcinoma cell; PC-3 (2 million cells) were injected on the upper half of the dorsal thorax of the mice. The tumors were allowed to grow for 6 weeks. The mice in the control group (no treatment) received 0.1 ml saline intraperitoneally for 5 days and the mice in the treated group received 50 mg/Kg fetuin. Treatment started the day after tumor inoculation for 5 days. Six week after the inoculation of tumor, mice were sacrificed and tumors were weighted. While tumors (average weight=325 mg) developed in 13 of 13 mice in the control group (treated with saline), none of the 9 mice treated with fetuin (50 mg/Kg) developed any tumor (Figure 14). Fetuin seems to completely inhibit the formation of prostate cancer (PC-3) in this experiment. Preliminary toxicological study showed that fetal fetuin has no toxicity in rats, mice and rabbits. No mortality was associated with high dose (250 mg/Kg) intraperitoneal administration of fetal fetuin in rats. Microscopic examination of the liver, kidney, spleen, lung and intestine of the mice treated with fetal fetuin (250 mg/Kg) showed no apparent abnormality of these organs. The I.V injection of the fetuin (250 mg/Kg) in rabbits did not result in any differences between the control group and experimental group in the following physiological parameters: body temperature, body weight, blood urea nitrogen, cholesterol levels, white blood cell count, hemoglobin and hematocrit and the sizes of spleen and thymus. All of these results suggest the low toxicity of fetal fetuin (data not shown). Fetal fetuin may therefore be developed as a anticancer agent.

Discussion

In the embryo, apoptosis (programmed cell death) is as essential as cell division and cell differentiation in properly regulating cell populations, organ formation and overall body shape (1-6). Due to the necessity of the above biological purposes, apoptosis is a highly active biological event occurring in embryo. Glucksmann has enumerated 74 separated examples of embryonic cell death in 1950 (7). The factor(s) that induce(s) the enumerous apoptosis event in embryo is unclear. To test the hypothesis that the highly active apoptosis event occurring in embryo is controlled by certain apoptosis-inducing proteins secreted by embryo, we tested whether apoptosis-inducing proteins can be isolated from embryo. We have screened the conditioned media of various cultured cell lines and found that 5 out of 7 cell lines derived from embryo contain apoptosis-inducing activity in the conditioned medium, while none of the conditioned media of 7 cell lines derived from adult tissues contains such an activity. Apoptosis-inducing activity is also found both in fresh embryo extract and in fetal serum. These results may support our hypothesis that embryo may secret apoptosis-inducing factors. To further characterize the apoptosis-inducing activity secreted by embryo, we have purified a 54 Kd apoptosis-inducing protein from the conditioned medium of C3H 10T1/2 cells (a cell line derived from mouse whole embryo), a 60 Kd protein from fresh mouse embryos and a 65 Kd protein from fetal bovine serum. The N-terminal sequence (19 amino acids) of the 54 Kd protein isolated from C3H 10T1/2 cells has been determined and was found not to match any known protein on BLAST serach. On the other hand, the apoptosis-inducing factor present in fetal bovine serum was identified to be fetuin---a fetal protein functions to control embryogenesis.

The identification of these two proteins enables us to study the embryonic apoptosis- inducing proteins on the molecular level. Strikingly, we found that these two proteins selectively induced apoptosis in cancer without affecting normal cell lines. For example, the 54 Kd protein isolated from the conditioned medium of C3H 10T1/2 cells induced apoptosis in 10 out of 12 cancer cell lines without affecting the 12 normal cell lines we tested. This characteristic was also found in fetal fetuin. Fetal fetuin induced apoptosis in cancer cell lines such as LNCaP (human metastatic prostate adenocarcinoma), PC-3 (human prostate adenocarcinoma), HL-60 (human promyelocyte leukemia), Colo 205 (human colon carcinoma) and Calu-1 (human lung carcinoma). Normal cell lines such as: CCD 39 Lu (human normal lung fibroblast), CCD 18 Co (human normal colon) and Wi-38 (human lung carcinoma), on the other hand, were not affected.

Fetuin was first identified over 5 decades ago (10). the present finding is the first report showing that fetuin may induce apoptosis. The main reason that the apoptosis-inducing activity of fetuin was not found before is that the biological activity of fetuin is largely affected by the method of preparation. For example, fetuin prepared by Zinc/Ethanol precipitation (Spiro method) is able to induce alkaline phosphatase but fails to inhibit trypsin (11). Whereas, fetuin prepared by Pedersen method (10) is able to inhibit trypsin without inducing alkaline phosphatase (11, 12). In the present study, we prepared the fetuin containing apoptosis- inducing activity by a modified Spiro method, which, unlike the original Spiro method, no prolonged dialysis nor chelating agent such as trisodium citrate is used. Hence, the fetuin we

prepared may remain in a zinc-charged form. Furthermore, the apoptosis-inducing activity of the fetuin we prepared can be further enhanced by incubation with high concentration of zinc, whereas incubation of barium completely abolished the apoptosis-inducing activity of fetuin (Figure 9). Theses results suggest that fetuin induced apoptosis in a zinc-dependent manner. The preparation of fetuin by Pedersen method (10) does not involve the treatment of zinc ion, hence, it is inactive in inducing apoptosis.

Fetuin is expressed at very high levels through out the long gestational period of bovine and accounts for up to 50% of the total fetal serum protein (13). However, the concentration of fetuin in sheep and bovine serum drastically reduce in adult to 1-2% of the fetal level (14). Although the quantitative change of fetuin during development seems to be obvious, the qualitative change (if any) of fetuin during development is unclear. It is known that enzyme such as gamma-glutamyltransferase changes its structure and activity during development. Depending on the developmental stage, the gamma-glutamyltransferase exists in two different types with different activity: a sialic acid-rich fetal type and a sialic acid-poor adult type (15). In the present study, we found that fetuin isolated from fetal serum strongly induced apoptosis, whereas the fetuin isolated from mature bovine serum is completely inactive. This result suggests the possible qualitative change of fetuin during development. Preliminary attemp to differentiate fetal fetuin from mature fetuin shows that they behave similarly on hydroxylapatite and anionic exchange chromatographies. However, the elution profiles of fetal and mature fetuins on a Con A-Sepharose chromatography were found to be significantly different (unpublished observation). This observation suggests that a developmental change on the glycosylated state of fetuin, which affects its apoptosis-inducing activity, may occur. the molecular difference between fetal and mature fetuins is currently under investigation.

Fetuins are members of the cystatin superfamily of proteins possessing two tandemly arranged cystatin domains and a third domain rich in proline and glycine (13). Secondary modifications, N-glycosylation, O-glycosylation, ser-phosphorylation, and proteolytic processing have all been described for a variety of fetuins from several species (16-19). Despite this wealth of information on the structure of fetuins, their biological function is still far from clear. Evidences suggest that fetuin may play an important role in embryonic development; during mouse embryogenesis, fetuin mRNA is expressed in a number of developing tissues and organs, including the heart, lung, kidney, nervous system and liver (14). In addition, fetuin mRNA is expressed in the developing limb buds of 12-day mouse embryos but not at 16 or 19 days of gestation (14). Furthermore, immunohistochemical study found that the colloid material (aggregate of dead cells) of developing human pituitary gland contains fetuin throughout the first half of gestation (20). This finding prompted the suggestion that fetuin is part of a homeostatic system, which

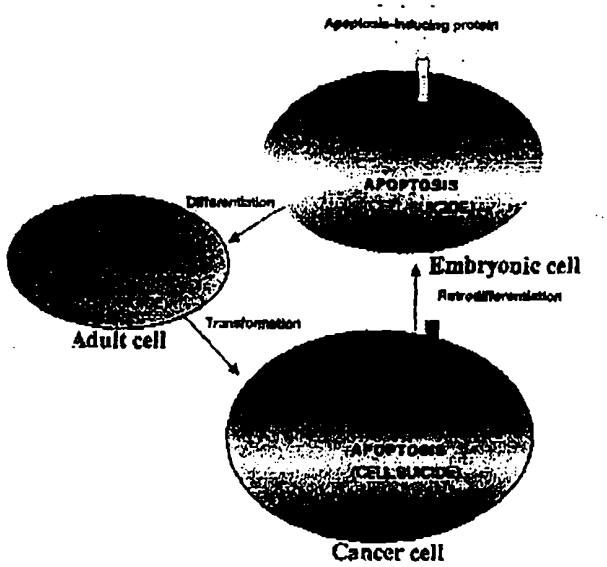


Figure 15. Hypothesis of "Retro-differentiational Apoptosis Cancer Therapy" The hypothesis is based on the following assumptions: Assumption 1--- embryonic cell (upper right) may contain all the machinery necessary for the development-related apoptosis which includes: extracellular apoptosis-inducing proteins, cellular surface receptors and the intracellular signal transduction cascade. Assumption 2--- Mature adult cell (left) may lose the machinery (e.g. receptor) necessary for the development-related apoptosis during the process of differentiation. Assumption 3--- Cancer cell (lower right), due to the retro- differentiational characteristic of malignancy, may regain the signal transduction machinery (e.g. receptor) for development-related apoptosis. Hence, the embryonic apoptosis-inducing proteins may selectively induce apoptosis in cancer without affecting normal cells and therefore may be developed as a novel class of anti-cancer agent.

controls remodelling and physiological cell death during development (20). In this regard, our current finding that fetuin contains apoptosis-inducing activity seems to consists with the conclusion made by this immunohistochemical study.

The two embryonic apoptosis-inducing proteins (the 54 Kd protein and the fetal fetuin) were found to induce apoptosis in cancer cell lines with LD 50s at 0.6-1 μM. These concentrations are about one thousand fold higher than concentrations of active molecules like TNF or TGF-beta required for biological effects. Including induction of apoptosis. Therefore, on the basis of the present analysis, a contaminant protein comprising 0.1 percent of the total protein and being active like a cytokine might cause the biological effect. Hence, the possibility that the apoptosis-inducing activity was caused by the minor undetectable components present in these protein preparations can not be ruled out. In this study, both highly enriched 54 Kd protein and fetal fetuin were used to determined the LD 50s. Although both proteins have been purified to be apparently

homogeneous and the bands shown are certainly the major dominating component, but it is not clear whether it exhibits the observed activity. The clarification of the true nature of the active molecule needs further work.

The availability of fetuin (an abundant fetal protein) enables us to test the effect of this embryonic apoptosis-inducing protein on animals. Fetal fetuin was therefore used as a model molecule to test the effect of these fetal apoptosis-inducing proteins on tumor animal models. We found that fetal fetuin showed strong anti-cancer activity in both P388 leukemia model and P- 3 prostate cancer model in mice. In the P388 leukemia model, we found that fetuin enhance survival rate by up to 141% in leukemia-bearing-mice. In PC-3 prostate cancer animal model, fetuin seems to completely inhibit the formation of prostate cancer in mice. Furthermore, Preliminary toxicological study showed that fetal fetuin has no toxicity in rats, mice and rabbits.

Based on these observations, we hypothesized that embryonic tissues may secret certain apoptosis-inducing proteins that selectively induce apoptosis in cancer without affecting normal cells. This group of fetal apoptosis-inducing proteins may contain anti-cancer activity in animals and may be developed as a novel class of anti-cancer agent.

The mechanism by which the 54 Kd protein and fetal fetuin selectively induced apoptosis in cancer without affecting normal cell is highly interesting. A working hypothesis has therefore been proposed to aid in the investigation of this observation. This working hypothesis is based on the following two well known biological phenomena: (1) apoptosis is a highly active biological event occurring in embryo and hence embryonic tissues may contain all the machinery necessary for the initiation of apoptosis. (2) the tendency of cancer cells to expressed antigens which are otherwise known to be normally expressed by embryonic, but not by adult cells is well known and was termed as "Retro-differentiation" (21). Based on these two well known biological phenomena, we hypothesized that, for the purpose of regulating cell populations, organ formation and overall body shape, embryo may secret certain apoptosis-inducing proteins functioning to initiate development-related apoptosis in certain embryonic tissues. Adult mature tissues, in which development-related apoptosis is not longer needed, may shut down the gene expression of the signal transduction machinery for development-related apoptosis and hence may not response to these apoptosis-inducing proteins. Cancer cells, on the other hand, due to the retro-differentiational characteristic of malignancy, may regain the signal transduction machinery for development-related apoptosis, and hence may be sensitive to these apoptosis-inducing proteins. Therefore, the apoptosis-inducing proteins secreted by embryo selectively induces apoptosis in cancer but not in normal cells. In other word, in this hypothesis, we proposed that the selectivity in apoptosis induction is due to the differential expression of the signal transduction machinery for apoptosis between adult cells and malignant cells. We further proposed that, among the mechanisms for adult cells to shut down the apoptotic machinery, turning off the gene expression of cell surface receptors for the embryonic apoptosis-inducing proteins may be the most feasible one, since shutting off the intracellular signal transduction pathway for apoptosis may also shut off other biological functions commonly using the same intracellular signal transduction pathway, which is not a favorite for natural selection. A good example falls in the case of NGF. It is known that as embryonic development proceeds, most neurons lost responsiveness to NGF. This is caused by the loss of NGF receptors during embryonic development (22). Hence it will be interesting to test whether the loss of the receptors of the embryonic apoptosis-inducing proteins on adult tissue results in the insensitivity of adult cells to these apoptosis-inducing proteins, and the re-expression of the receptors on cancer cells cause the sensitivity of cancer cells to the apoptosis-inducing proteins. Furthermore, as we have found in this study, each apoptosis-inducing protein seems to specifically induce apoptosis in certain type of cancers. For example, the 54 Kd protein induced apoptosis in LNCaP, MCF-7 and HL-60 without affecting CaLu-1 cells and the 60 Kd protein isolated from fresh mouse embryo induced apoptosis in HL-60 without affecting LNCaP and MCF-7 cells, whereas fetal fetuin induced apoptosis on all of the above cell lines. This finding may suggest that different cancer may express different receptors for the embryonic apoptosis- inducing proteins. The receptors for the 54 Kd apoptosis-inducing protein and fetal fetuin are currently under investigation in our laboratory.

Due to the retro-differentiational characteristic of malignant cells, the embryonic apoptosis-inducing proteins may selectively induce apoptosis in cancer without affecting normal cells and therefore may be developed as a novel class of anti-cancer agent. This new concept (Figure 15) may constitute a new approach for cancer therapy, which we tentatively designated as "Retro-differentiational Apoptosis Cancer Therapy" (R-ACT).

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